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THE ETIOLOGY OF COLORADO TICK FEVER

By LLOYD FLORIO M.D., MABEL O STEWART AND EDWARD R. MUGRAGE, M D

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(Received for publication, June 15 1945)

Attempts to isolate the causative agent of Colorado tick fever have been unsuccessful (1) but it has been maintained in both man and hamster by serial passage (2) Since we have never been able to visualize microscopically the infectious agent of this disease either in man or in hamsters when we knew that the etiologic agent was present in the serum, it seemed probable we were dealing with a virus and therefore ultrafiltration experiments were tried

Methods and Materials

Two strains[1] of Colorado tick fever were used. Pooled serum from infected hamsters was kept frozen in the ice compartment of an electric refrigerator until used. The serum was thawed, diluted with three parts of sterile physiological saline, and immediately filtered through a Seitz EK filter under negative pressure of 15 cm of mercury A portion of this filtrate was saved as a control, and the remainder filtered through gradacol membranes[2] with positive pressure which never exceeded 100 cm of mercury This maximum pressure was used only with the 24 mμ membrane filtration experiments. In every instance in which serum was filtered through membranes of this pore size the material was first passed through either a 262 or 181 mμ membrane. No filtration required more than 7 hours. All membranes were tested for major defects with *B prodigiosus* at the termination of each experiment. None were found

Hamsters in groups were each given ½ cc. intraperitoneally of the control serum or filtrate. Four days after the injection the animals were bled to death from the heart (3) and autopsied immediately[3] A small portion of the blood was oxalated for study and the remainder allowed to clot. The pooled serum from some of the hamsters was used to inoculate new groups of animals, and also human volunteers as seen in the sequence of transfers depicted in Text fig 1

EXPERIMENTAL RESULTS

The control animal groups were infected in every instance We used the lowered white blood cell count[3] as evidence of having successfully infected

[1] One strain had been procured from a natural instance of the disease and had been previously carried through 7 serial hamster passages. The other strain came from a natural instance of the disease and had been carried through 4 volunteers, then serially through 7 hamster groups another volunteer and finally serially through 7 more hamster groups.

[2] These membranes were prepared and calibrated by Walter Mack of the Hooper Institute for Medical Research of the University of California School of Medicine according to the method described by Bauer J H. and Hughes T P *J Gen Physiol.*, 1934, 18, 143

[3] A study of the histologic picture is in progress

the hamsters studied Since the cytoplasmic bodies in lymphocytes are an inconstant finding in infected animals, they were not used as a criterion of infection Sera from both strains were filtered through 262 and 181 mμ membranes All of the animal groups injected with these membrane filtrates were infected

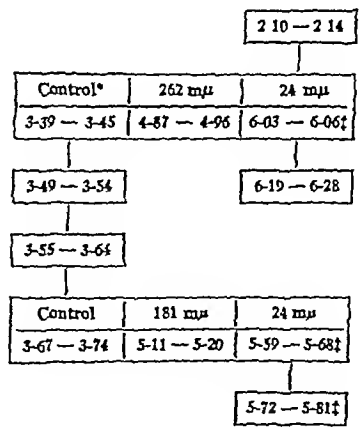
Seven separate filtration experiments were carried out using 24 mμ membranes Two of the 7 initial groups of injected hamsters showed clear cut evidence of infection On further animal passage, infection was demonstrated in 3 more groups There was one failure with each of the 2 strains

Table I shows the mean white blood cell count for each group of hamsters, as obtained at time of death, and the number of animals in each group, which showed bodies in the lymphocytes, regardless of whether or not the hamsters were utilized in the serum pool used to inoculate the next group of animals

Since the white blood cell count is not influenced by the age or sex (3) mention of them has been omitted from the table There was approximately an equal number of males and females ranging from 2 to 9 months of age

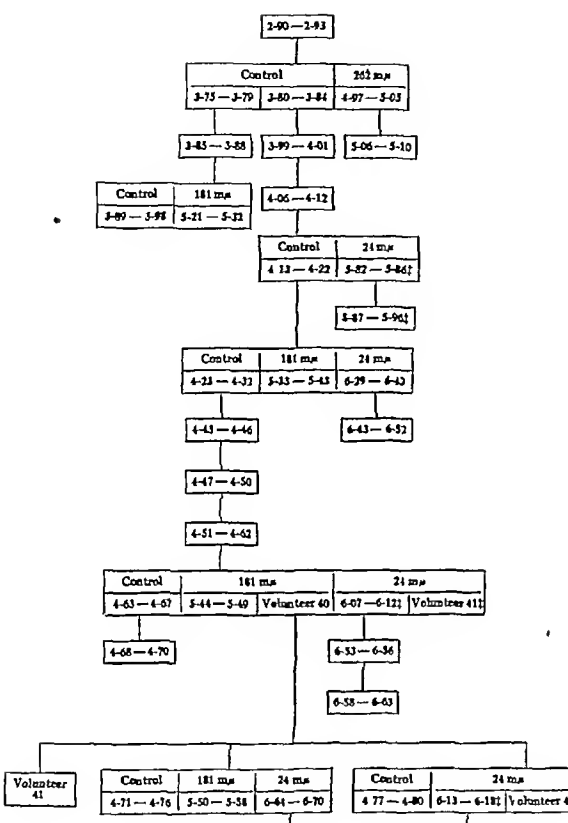
The standard error was determined for the infected hamsters inoculated with the control sera and with the filtrates from the 262, 181, and 24 mμ membranes respectively Since the difference between the means of these groups was always less than two standard deviations, the data were combined The hamster groups inoculated with the 24 mμ membrane filtrates that gave no evidence of infection are not included

Human volunteers were used as a further check on the results obtained in hamsters



* Everywhere in this text figure the numbers are inclusive.
‡ No evident infection

TEXT-FIG 1 Sequence of transfers of two strains of Colorado tick fever through hamsters and human volunteers



TEXT FIG 1—*Concluded*

Pooled serum from the fifteenth hamster transfer (Text fig 1, animals 4–51 to 4–62) was passed through a 181 mμ membrane and 8 cc. injected subcutaneously into volunteer 40 Hamsters 5–44 to 5–49 were each inoculated intraperitoneally with ½ cc of this same filtrate

TABLE I

Mean White Blood Cell Counts and the Presence of Cytoplasmic Bodies in the Lymphocytes in Hamsters Inoculated with Control and Gradacol Membrane—Filtered Colorado Tick Fever Serum

Animal Nos	Source of serum	Age of serum	Mean white blood cell count in thousands per c. mm	No of animals with cytoplasmic bodies in lymphocytes (100 cell hemogram)
		Control groups		
		days		
3-39 — 3-48*	2-10 — 2-14	8	5 22	5
3-49 — 3-54	3 39 — 3-45	10	4 08	4
3-55 — 3-64	3-49 — 3-54	12	4 05	4
3-65 — 3 74	3 55 — 3-64	1	5 42	6
3-75 — 3 79	2 90 — 2-93	7	3 04	1
3-80 — 3-84	2-90 — 2-93	7	9 31	5
3-85 — 3-88	3-75 — 3 79	15	4 40	3
3-89 — 3-98	3-85 — 3-88	1	6 26	5
3-99 — 4-02	3-80 — 3-84	25	5 23	2
4-03 — 4-12	3-99 — 4-01	12	4 43	4
4-13 — 4-22	4-06 — 4-12	5	5 59	6
4-23 — 4-42	4-13 — 4-22	2	4 56	7
4-43 — 4-46	4-23 — 4-32	13	4 80	2
4-47 — 4-50	4-43 — 4-46	14	4 23	2
4-51 — 4-62	4-47 — 4-50	7	4 05	6
4-63 — 4-67	4-51 — 4-62	1	3 87	2
4-68 — 4-70	4-63 — 4-67	15	4 41	2
4-71 — 4-76	Volunteer 40	5	4 25	1
4-77 — 4-80	Volunteer 40	13	3 16	0
4-81 — 4-86	Volunteer 42	16	4 26	2
Mean			4 77	
Standard deviation			2 08	
		262 mμ membrane filtration groups		
		days		
4-87 — 4-96	2-10 — 2-14	8	3 98	3
4-97 — 5-05	2 90 — 2-93	7	5 23	8
5-06 — 5-10	4-97 — 5-05	25	8 40	2
Mean			5 43	
Standard deviation			2 96	

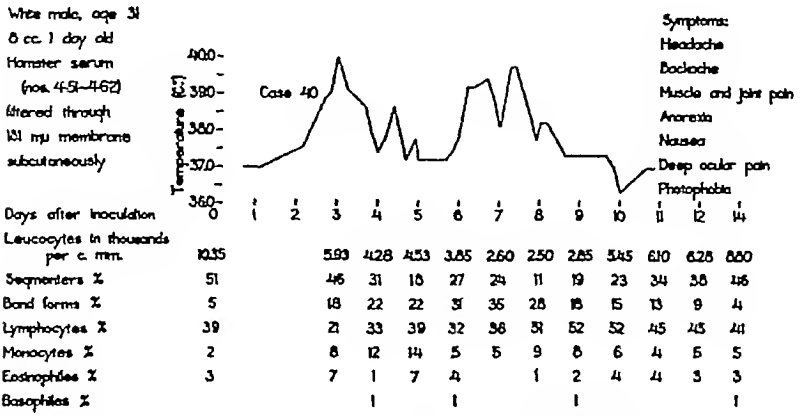
* The numbers are inclusive

TABLE I—*Concluded*

Animal Nos.	Source of serum	Age of serum	Mean white blood cell count in thousands per c. mm.	No. of animals with cytoplasmic bodies in lymphocytes (100 cell hemogram)
181 mμ membrane filtration groups				
		days		
5-11 — 5-20	3-55 — 3-64	1	3 71	4
5-21 — 5-32	3-85 — 3-88	1	7 21	4
5-33 — 5-43	4-13 — 4-22	2	6 47	2
5-44 — 5-49	4-51 — 4-62	1	3 71	4
5-50 — 5-58	Volunteer 40	5	4 79	3
Mean			5 34	
Standard deviation			2 99	
24 mμ membrane filtration groups (no evident infection)				
		days		
5-59 — 5-71	3-55 — 3-64	1	6 43	1
5-72 — 5-81	5-59 — 5-68	2	6 40	0
5-82 — 5-86	4-06 — 4-12	5	7 23	0
5-87 — 5-96	5-82 — 5-86	7	7 25	1
24 mμ membrane filtration groups (no apparent infection in initial hamster groups)				
5-97 — 6-06	2 10 — 2 14	8	6 49	0
6-07 — 6-12	4-51 — 4-62	1	7 07	0
6-13 — 6-18	Volunteer 40	13	7 10	0
24 mμ membrane filtration groups (infected)				
6-19 — 6-28	6-03 — 6-06	6	4 01	5
6-29 — 6-42	4-13 — 4-22	2	4 75	4
6-43 — 6-52	6-29 — 6-42	9	4 69	2
6-53 — 6-57	6-07 — 6-12	1	5 23	0
6-58 — 6-63	6-53 — 6-56	1	3 05	4
6-64 — 6-70	Volunteer 40	5	4 16	0
6-71 — 6-77	6-64 — 6-70	48	3 94	0
6-78 — 6-83	6-13 — 6-18	1	3 72	1
Mean (24 mμ membrane filtration groups, infected)			4 30	
Standard deviation			1 73	
280 infected animals				
Mean			4 81	
Standard deviation			2 30	

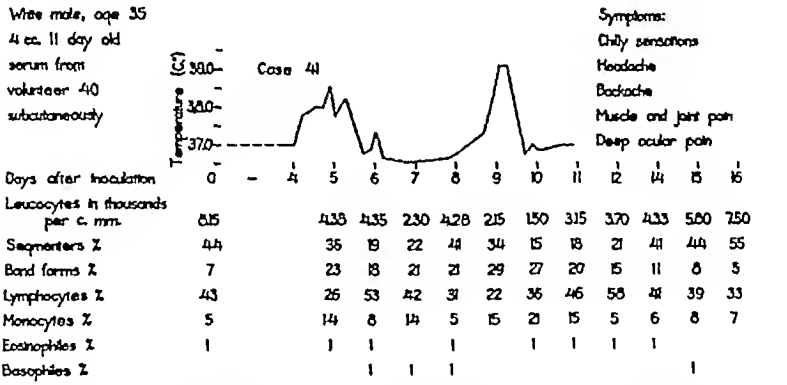
Both the volunteer and the animals showed typical infection Text-fig 2 depicts the course of the disease in volunteer 40

Case 40 and all subsequent volunteers were physically healthy institutional patients, confined in a separate building for at least 1 month prior to inoculation



Days after inoculation	0	1	2	3	4	5	6	7	8	9	10	11	12	14
Leucocytes in thousands per c. mm.	10.35			5.93	4.28	4.53	3.85	2.60	2.50	2.85	3.45	6.10	6.28	8.80
Segmenters %	51			46	31	18	27	24	11	19	23	34	38	46
Band forms %	5			18	22	22	31	35	28	16	15	13	9	4
Lymphocytes %	39			21	33	39	32	36	51	52	52	45	45	41
Monocytes %	2			8	12	14	5	5	9	8	6	4	5	5
Eosinophiles %	3			7	1	7	4		1	2	4	4	3	3
Basophiles %					1		1			1				1

TEXT-FIG 2 Colorado tick fever in volunteer 40 inoculated with 181 mμ membrane filtrate from the fifteenth hamster transfer



Days after inoculation	0	–	4	5	6	7	8	9	10	11	12	14	15	16
Leucocytes in thousands per c. mm.	8.15			4.38	4.35	2.30	4.28	2.15	1.50	3.15	3.70	4.33	5.80	7.50
Segmenters %	44			35	19	22	44	34	15	18	21	41	44	55
Band forms %	7			23	18	21	21	29	27	20	15	11	8	5
Lymphocytes %	43			26	53	42	31	22	36	46	58	41	39	33
Monocytes %	5			14	8	14	5	15	21	15	5	6	8	7
Eosinophiles %	1			1	1		1		1	1	1	1		
Basophiles %					1	1	1						1	

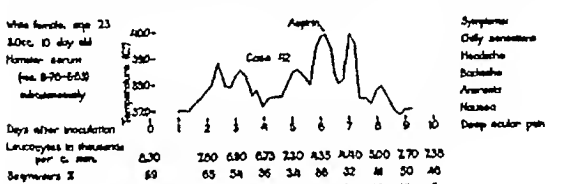
TEXT-FIG 3 Colorado tick fever in volunteer 41 after a previous unsuccessful attempt to infect with 24 mμ membrane filtrate

A portion of the material filtered through the 181 mμ membrane and used to infect volunteer 40 was filtered through a 24 mμ membrane Sixteen cc of this filtrate was injected subcutaneously into volunteer 41, and ½ cc intraperitoneally into each of 6 hamsters Only the hamsters (Nos 6–53 to 6–63) of 2 subsequent transfers showed typical infection Since volunteer 41, who remained well for 15 days, had previously lived in an endemic area, we tested his immunity by inoculating him subcutaneously with 4 cc. of undiluted serum from volunteer 40 He developed Colorado tick fever as seen in Text-fig 3

A further filtration experiment was tried to see whether similar results could be obtained by using human instead of hamster serum

Serum from volunteer 40 was prepared in the same way as the hamster material, then filtered through a 181 mμ and finally through a 24 mμ membrane Hamster groups injected with the control and 181 mμ material showed positive findings Volunteer 42 received subcutaneously 16 cc. of the 24 mμ filtered serum and remained well for 32 days. The animal groups given the same 24 mμ filtrate were found to be infected on second transfer (6–78 to 6–83) Three cc. of this pooled undiluted hamster serum (6–78 to 6–83) was given subcutaneously to volunteer 42. Two days later she developed typical Colorado tick fever as seen in Text fig 4

There remain the possibilities that hamster serum on inoculation into human beings may give a clinical picture similar to Colorado tick fever or



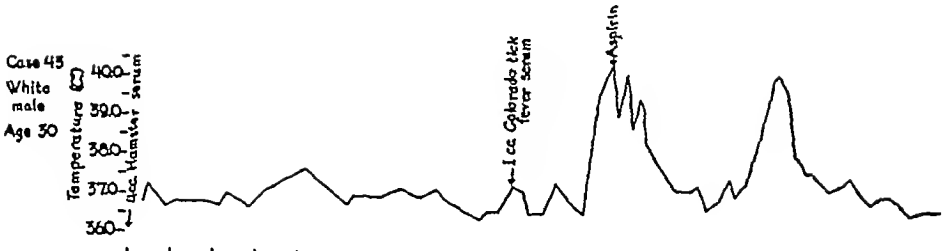
TEXT FIG 4 Colorado tick fever in volunteer 42 injected with serum from hamsters inoculated with a 24 mμ membrane filtrate, which filtrate had failed to cause infection when injected into this volunteer

that in our series of transfers we may have picked up an extraneous agent from presumably normal hamsters that would give a lowered white blood cell count in the animals and a clinical picture resembling Colorado tick fever in human beings These possibilities were eliminated in the following way—

Five hamsters were bled to death and ½ cc. of the pooled serum inoculated into each of 10 animals. They were bled after 4 days and the process repeated serially through 10 groups of approximately 10 animals each. The hamsters were selected at random from our colony and included males and females of various ages above 2 months as well as a few pregnant animals. The mean white blood cell count of the initial 5 animals was 7,975 and for the 10 subsequent groups of 101 animals 7,651 ± 1 686 with a mean range of 6,207 to 8,218 The mean for this group of 101 hamsters is within two standard deviations of the mean 8,088 ± 1 776 previously established for 114 normal animals (3)

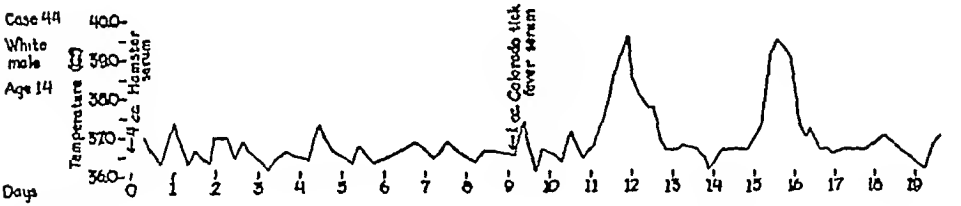
Cytoplasmic bodies in lymphocytes were found in 2 animals on the sixth transfer only

Volunteers 43 and 44 were each inoculated subcutaneously with 4 cc. of hamster serum from the final serial transfer For 9 days these volunteers showed neither a significant elevation of



Days	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Leucocytes in thousands per c. mm.	615		750		665			615		660		735	375		360	395		155		568
Segmenters %	48		41		55			55		52		50	46		21	59		22		32
Band forms %	4		4		4			4		6		22	20		16	56		29		11
Lymphocytes %	37		49		33			30		35		20	29		52	19		43		49
Monocytes %	10		5		6			6		6		6	5		8	4		5		7
Eosinophiles %	1		1		1			3		1		1			3	1		1		1
Basophiles %					1			2				1				1				

TEXT-FIG 5 Colorado tick fever in volunteer 43 injected with serum from a natural instance of the disease 9 days after a previous inoculation with normal hamster serum of the tenth serial transfer



Days	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Leucocytes in thousands per c. mm.	775		775		730			740		748		670	420		370	310		365		610
Segmenters %	41		24		42			58		47		67	47		28	42		21		31
Band forms %	3		2		3			2		3		16	29		18	30		18		6
Lymphocytes %	49		69		46			34		40		10	22		45	22		56		58
Monocytes %	4		2		5			3		6		7	7		8	5		4		3
Eosinophiles %	3		3		4			2		4					1	1		1		

TEXT-FIG 6 Colorado tick fever in volunteer 44 injected with serum from a natural instance of the disease 9 days after a previous inoculation with normal hamster serum of the tenth serial transfer

temperature nor a depression of the leucocyte count. They were then inoculated intravenously with 1 cc of serum from a natural instance of Colorado tick fever Both developed typical Colorado tick fever 2 days later The findings are depicted in Text figs 5 and 6

Volunteers 41, 42, and 44 showed typical lymphocytic inclusion bodies during convalescence as described in a previous paper (2)

DISCUSSION

Although the original hamster groups into which 24 mμ membrane filtrate was injected usually failed to show clear cut evidence of infection, an additional transfer or two evidently caused a multiplication of the infectious agent to the point that characteristic white blood cell counts were obtained

The 24 mμ membranes did not permit the passage of the infectious agent of Colorado tick fever in *sufficient quantity to infect two susceptible human* beings in the dose given In one instance, when the 24 mμ filtrate was injected into a volunteer and a group of hamsters, the human being was not infected, but the hamsters manifested the disease on second transfer The serum from this second transfer was then injected into the same volunteer and caused typical Colorado tick fever

In a previous paper (2) we reported inclusion bodies in the hamster lymphocytes in those animals infected with Colorado tick fever We had never seen these inclusions in the blood of normal animals However, these bodies have now been seen in two animals that were inoculated with 24 mμ membrane filtrate which did not manifest disease (Table I), as well as in two animals of one group in our transfer of normal hamster serum through 10 serial passages Thus lymphocytic inclusion bodies have been demonstrated in 4 out of 258 animals which were either normal or which failed to develop the tick fever These findings argue against the specificity of these bodies They remain diagnostically important, however, for they were found in 115 out of 280 animals in this series which came down with the fever, a significantly high incidence We now feel that the lymphocytic inclusion bodies found in human beings during convalescence from Colorado tick fever are also not wholly specific since we have seen similar appearing structures in other diseases

Experiments carried out on volunteers 43 and 44 eliminate the possibility that hamster serum obtained from serial animal passages when inoculated into human beings will result in signs and symptoms of Colorado tick fever due either to the hamster serum itself or to some extraneous agent picked up during the serial passages

Elford (4) points out that the size of a particle filtered through a gradacol membrane is smaller than the average pore diameter of the membrane used The infectious agent of Colorado tick fever is partially retained by the 24 mμ membrane We estimate the particle size to be approximately 10 mμ. This makes the causative agent of Colorado tick fever one of the smallest known

SUMMARY AND CONCLUSIONS

1 The infectious agent of Colorado tick fever filtered through 181 mμ membranes caused infection in hamsters and one volunteer

highly purified preparations of this virus have been obtained only recently, and these from the allantoic fluid of infected chick embryos (7, 8) Such preparations are tremendously active and are composed of uniform particles about 100 mμ in diameter which possess distinctive physical, chemical, and biological properties (7–13) The present investigation was undertaken in the hope of obtaining highly purified preparations of influenza virus from infected mouse lungs for comparison with the product isolated from infectious allantoic fluid It is apparent that infected mouse lungs represent a starting material so different from the allantoic fluid of infected chick embryos that the isolation from each source of a substance possessing common distinctive properties would constitute strong evidence that the substance is the virus

Methods

Red Cell Agglutination —Quantitative red cell agglutination tests were made by the method of Hirst and Pickels (14) as modified for use in this laboratory (15) A purified virus standard was used throughout, hence the agglutination titers reported herein are on a comparable basis

Infectivity Titrations —Titrations in chick embryos and in mice were carried out by previously described methods, using 10 embryos and 10 mice for each dilution tested (16, 17)

EXPERIMENTAL

Inoculation of Mice —The original inoculum used in this study was a mouse adapted strain of PR8 influenza virus kindly provided by Dr G K. Hirst Serial passage of the virus provided additional material for inocula Four week-old white mice from the colony of the Department of Animal and Plant Pathology of the Institute were used throughout the investigation

In an effort to increase the red cell agglutinating titers of the lung tissue extracts, a method of inoculation by spraying was compared with the standard technique of intranasal instillation of droplets under ether anesthesia Typical results are represented by the following experiment —

Forty 4-week-old mice were divided into 2 groups of 20 mice. Each mouse in one group was inoculated under ether anesthesia with 2 drops (*ca* 0 05 ml) of a 1 per cent suspension of infected mouse lung in 0 1 M phosphate buffer at pH 7 The other group was placed in a glass chamber equipped with a loose fitting wire and cardboard top and sprayed with the 1 per cent suspension of infected mouse lung for a period of 5 minutes [1] The vaporizer produced an extremely fine and persistent mist some of which was naturally inhaled by the mice as they moved about in the glass chamber It was operated from a compressed air line and, at a pressure sufficient to produce an appreciable fog, used from 2 to 3 ml of 1 per cent suspension during the 5 minute period of spraying Groups of 5 mice from each of the 2 lots were sacrificed at 24 hour intervals, with the exception that at the later periods, particularly at 96 hours, there were less than 5 because some animals had died with typical consolidation of the lungs The lungs were perfused before harvesting, as described below, and were ground in a mortar with sterile sand and enough 0 1 M phosphate buffer at pH 7 to give a 10 per cent suspension on the basis of the wet weight of the lungs Coarse particles and sand were removed by centrifugation at 5,000 R.P M. for about 5 minutes The supernatant fluids

[1] The sprayer employed was a Vaponefrin vaporizer produced by the Vaponefrin Company of 6812 Market Street, Upper Darby, Pennsylvania

were then tested for ability to agglutinate red cells by the procedure referred to under Methods. The agglutination titers (CCA activities) of the 10 per cent suspensions from the mice inoculated by the standard droplet method were 60, 87 and 43 standard CCA units per ml at 24 48 and 72 hours, respectively Those of the sprayed mice were <4 76 149 and 67 CCA units per ml at 24 48, 72 and 96 hours, respectively

In general it was observed that the agglutination titers declined as the lungs became more than 50 per cent consolidated. The titers of the suspensions from completely consolidated lungs were most erratic, for some had appreciable titers and others were inactive It was apparent from the results of the above and of several other tests that, under the conditions used the maximum concentrations of extractable virus (as indicated by CCA activity) occurred at approximately 48 hours in the case of the mice inoculated by the standard method and at approximately 72 hours in the case of sprayed mice. More important, the maximum titers of the lung suspensions from sprayed mice were almost invariably about 50 per cent higher than those of the mice inoculated by intranasal instillation of droplets.

Method of Harvesting Lungs.—Influenza virus is adsorbed on red cells of various species (18–21) Moreover both normal and immune sera inhibit the agglutination of red cells by virus (22) Since as will be described later adsorption of virus on and agglutination of chicken red cells became a necessary step in the purification procedure, it appeared desirable that as much blood as possible be eliminated from the mouse lungs in order to achieve a maximum agglutination titer This procedure received justification when the agglutinating titers of suspensions obtained from perfused lungs were compared with those from unperfused lungs. In the perfusion process, the mouse was put under deep ether anesthesia and the thoracic cavity was opened quickly A 20 gauge needle connected by rubber tubing to an elevated reservoir of saline was inserted in the right ventricle of the heart and an incision was made in the left ventricle Saline was allowed to flow through the lungs and out of the heart for about 15 seconds By this time the lungs were white except for areas of consolidation With the perfusing needle still inserted in the heart, the latter was jerked out by means of forceps with a quick movement which left the lungs behind. The lungs were then extricated with forceps, generally in one cluster, and placed in a Petri dish containing 0 1 M phosphate buffer at pH 7 The buffer which served to keep the lungs moist and to wash off surface blood was removed at the end of the harvest by placing the lungs on filter paper on a Büchner funnel and applying gentle suction. The lungs were weighed and then ground and the clarified suspensions in phosphate buffer were tested for CCA activity In 2 experiments, the 10 per cent suspensions of unperfused lungs titered 50 and 73 CCA units per ml while corresponding suspensions of perfused lungs gave values of 106 and 117 The titers obtained later from the perfused lungs of groups of 150 to 200 mice were consistently in the neighborhood of 120 CCA units per ml of 10 per cent suspension The highest titer observed for a large group of mice was 225 CCA units per ml or 2,250 units per gm of wet lung

Comminution of Lungs.—It is convenient to grind small lots of lungs in a mortar with sterile sand and buffer With 20 to 40 gm lots this procedure is too laborious hence tests were made to see whether a Waring blendor might be used It was found that despite an appreciable amount of foaming in the blendor the infectivities and CCA activities of the material thus ground were the same as those of the material ground in a mortar Thereafter the blendor was employed to release virus particles from lung tissue. The lungs were ground for 2 minutes in 9 times their weight of 0 1 M phosphate buffer at pH 7 and the resulting 10 per cent suspension was spun in an angle centrifuge at 5,000 R.P.M. for 3 to 5 minutes to remove gross particles.

Storage of Virus and Choice of Suspending Medium.—In most cases during the present investigation it was not possible to work through a sufficiently large number of mice at one time to enable an immediate isolation of virus to be made from the lung material Therefore

it was necessary to store 200 to 400 ml lots of 10 per cent suspension until an adequate amount had been accumulated The experiments of Horsfall indicated that 20 per cent suspensions of infected mouse lungs in infusion broth containing horse serum could be stored at dry ice temperature and frozen and thawed repeatedly without appreciable alteration in the infectious quality of the virus (23) Investigations with PR8 virus from allantoic fluid indicated that 0 1 M phosphate buffer at pH 7 is a good medium in which to preserve the virus at 4° C (24) and that it also maintains a constant CCA activity at −70° C for at least a month (15) Therefore, lung suspensions were made in 0 1 M phosphate buffer at pH 7 as described above and were stored at −70° C for periods up to 1 month With regard to the storage of purified or partially purified virus, it was found that the CCA activity of a preparation of mouse lung virus partially purified by differential centrifugation remained constant for at least 10 days in 0 1 M phosphate buffer at pH 7 at 4° C On the other hand, more than 50 per cent of the CCA activity was lost in the same period of time when the material was stored in either water or in a composite phosphate-glycine-NaCl buffer (25)

Concentration and Purification of Virus—Frozen 10 per cent suspensions of infected mouse lung were thawed and pooled The freezing and thawing process always resulted in the formation of an amount of aggregated insoluble material equivalent to about 15 per cent of the total nitrogen When this material was removed by low speed centrifugation and suspended in phosphate buffer, it was found to possess on a nitrogen basis only 15 per cent of the CCA activity possessed by the supernatant fluid It was apparent that the bulk of the aggregated material was non-viral in character and it was found that a substantial amount of the CCA activity associated with it could be recovered by one extraction with 0 1 M phosphate buffer

Several attempts were made to obtain the virus from clarified, thawed suspensions by centrifugal methods such as had previously been used by other investigators (26, 27) However, it was found that such preparations, of which No 2B in Table I is an example, invariably had low agglutination and infectious titers, possessed a poor solubility in 0 1 M phosphate buffer, were very inhomogeneous in the analytical ultracentrifuge and were manifestly composed to a large extent of non viral lung material Therefore, it was necessary to find some other method for purification of the virus

It was observed that normal lung material, like the normal protein from allantoic fluid (10), was not adsorbed on chicken red cells. It seemed probable, therefore, that much of the inactive material might be left behind if the virus were adsorbed on and subsequently eluted from chicken red cells (18, 19, 21, 28) This proved to be the case and combined with the procedure of differential centrifugation furnished a method for obtaining highly purified virus preparations The final method will be given in detail at the end of this section The CCA activities and infectivities of preparations obtained by various procedures are summarized in Table I

In the course of the study, some differences between the non-viral materials of mouse lungs and of allantoic fluid emerged which have an important bearing on the purification of virus from mouse lungs The sedimentable protein of normal allantoic fluid is not appreciably affected by freezing and thawing and is readily soluble in 0 1 M phosphate buffer (10) Since both of these properties are also those of the infectious material, they provide no advantage in the purification of the virus On the other hand, a substantial amount of the sedimentable non-viral material from mouse lungs is aggregated by freezing and thawing, and both this material and the unaggregated material isolated from normal lungs by differential centrifugation are poorly soluble in 0 1 M

phosphate buffer It appears from a comparison of the CCA activity of preparation 3 in Table I with those of some of the other preparations that the freezing and thawing of the mouse lung suspensions, presumably by virtue of eliminating some of the non viral material, is a valuable aid in separating the virus from the balance of inert material The CCA activities of 4 other preparations were 5 to 6 times greater than that of preparation 3 and the latter was the only one made from a freshly prepared, unfrozen 10 per cent suspension It was found possible, however, to increase greatly the CCA

TABLE I

The Activity of Preparations of PR8 Influenza Virus Obtained from Infected Mouse Lungs

Preparation No	Method of preparation	CCA units per mg of nitrogen	50 per cent end-points in grams of nitrogen	
			Embryos	Mice
1	2 adsorptions on and elutions from red cells and 2 cycles of differential centrifugation	28 400	$10^{-11.3}$	$10^{-11.8}$
2A	Same as 1	24 600	$10^{-11.3}$	$10^{-11.6}$
2B	Same starting material as 2A 3 cycles of differential centrifugation	2 080	$10^{-10.4}$	$10^{-9.4}$
3	Same as 1†	5 040	Not tested	$10^{-11.7}$
4	1 adsorption on and elution from red cells and 3 cycles of differential centrifugation	33 000	Not tested	Not tested
5	Same as 4	29 800	$10^{-11.4}$	$10^{-11.3}$

* Infectivity end-points in the case of the embryo tests and weighted end points in the mouse tests. In both cases 10 animals were used per test dilution See references 16 and 17

† Starting material, however was freshly prepared unfrozen 10 per cent suspension whereas in 1 and in all other cases the starting material had been stored at −70 C and thawed just before use

activity of preparation 3 on the basis of the behavior of normal lung material in 0 1 M phosphate buffer

It was observed that highly purified preparations such as 1 and 2A in Table I, were, like comparable preparations from allantoic fluid, readily soluble in 0 1 M phosphate buffer and that very little sediment formed upon storage at 4°C for periods up to 1 month In marked contrast preparations obtained by differential centrifugation alone and ones obtained from unfrozen suspensions by a combination of methods (see preparations 2B and 3 in Table I) constantly deposit aggregates upon storage at 4°C. If these aggregates are removed by centrifugation, the CCA titer of the supernatant fluid rises For example, when preparation 3 was subjected to this treatment, the CCA activities per mg of nitrogen of the material remaining suspended in phosphate buffer were

5,040, 10,500, 14,000 and 18,400 when freshly isolated and after 5, 8, and 16 days, respectively Such a spectacular increase in CCA activity has not been observed in the case of the less pure preparations obtained by centrifugation alone and the highest titer obtained with these was 3,100 CCA units per mg of nitrogen Hence, it appears that the possibility of separating non-viral lung material from infectious material is at least in part dependent upon the ratio of the concentrations of each This principle was utilized in obtaining preparations 4 and 5 In these instances, one adsorption on and elution from red cells was used to separate the infectious particles from the bulk of the inert lung material A small amount of the latter is brought along mechanically in this process due to the tendency of the material to aggregate during centrifugal manipulations in 0 1 M phosphate buffer This same property, however, makes possible the removal of the inert material from the preparation, for, after 3 cycles of differential centrifugation, the preparation is highly active and shows the physical characteristics of the material obtained by 2 adsorptions on and elutions from red cells

The complete procedure for the isolation of PR8 virus from infected mouse lungs is given in the following protocol which is exemplary of the combination of methods found to yield the best results The example used is preparation 5 of Table I

Four-week-old white mice from the colony of the Institute were sprayed in a glass chamber in lots of 20 for 5 minutes with a 1 per cent clarified suspension of infected mouse lung in 0 1 M phosphate buffer at pH 7 After about 72 hours, when 10 to 20 per cent of the mice had succumbed to the disease, the surviving animals, most of which were moribund, were placed individually under deep ether anesthesia and their lungs were perfused with 0 85 per cent saline and removed and placed in 0 1 M phosphate buffer at pH 7 at room temperature (20–25° C) At the end of the harvest, the buffer was removed by placing the lungs on filter paper on a Büchner funnel and applying gentle suction The moist lungs were quickly weighed on a rough balance and ground in a Waring blendor for 2 minutes with enough phosphate buffer to give a 10 per cent suspension The material from the blendor was clarified by spinning for 5 minutes at 5,000 R.P M. in an angle centrifuge and by pouring the supernatant fluid through glass wool to remove the small amount of fluffy material which did not sediment. The clarified suspension was stored at −70° C in sterile, stoppered Lusteroid tubes until the lungs had been harvested from about 1,200 mice The accumulated frozen suspensions were liquefied by immersion of the tubes in lukewarm water until the ice was just thawed but the fluid was still cold The pooled thawed suspension was centrifuged at 4° at 3,500 R.P M for 5 minutes The pellets obtained by this centrifugation were extracted once with a total of 80 ml of cold buffer and the extract was added to the clarified suspension To 1,880 ml of cold clarified suspension possessing 116 standard CCA units and 1 2 mg of nitrogen per ml were added 38 ml of packed chicken red cells (15) After swirling thoroughly, the mixture was allowed to stand at 4° C for 2 hours At this time the agglutinated cells had settled to the bottom of the flask and most of the supernatant fluid, which possessed about 7 CCA units per ml, was removed by decantation The remaining supernatant fluid was separated from the cells by centrifuging the mixture at 3,500 R P M for 3 minutes and decanting The cells were then suspended in 150 ml of phosphate buffer and the virus was eluted by incubating

at 37° C. for 90 minutes with occasional swirling. The red cells were removed from the mixture by centrifuging at 5,000 R.P.M. for 5 minutes. The virus was recovered from the pink-colored eluate by centrifugation in the quantity centrifuge at 24 000 R.P.M. for 15 minutes. The pellets, which contained the virus, were suspended in 25 ml. of phosphate buffer and spun in the angle centrifuge at 5,000 R.P.M. for 5 minutes. The process of alternate high- and low-speed centrifugation was repeated 2 additional times to yield about 24 mg. of purified material which readily dissolved in 10 ml. of 0.1 M phosphate buffer at pH 7 to give a bluish-white opalescent solution.

Properties of Purified Mouse Lung Virus

Biological Properties.—For the sake of comparing infectious qualities, preparations of PR8 virus were twice obtained from allantoic fluid at the same time that preparations of mouse virus were being isolated and under identical conditions. The results were interesting in that they indicated that the purified mouse virus preparations, though containing virus highly adapted to mice, were nevertheless as infectious in embryos as were comparable preparations of egg adapted virus. The reverse was not true, however, for the purified preparations of mouse virus were about 100 times as infectious for mice as the exactly corresponding preparations of virus from allantoic fluid.[2] The preparations obtained from allantoic fluid, which corresponded to mouse virus preparations 2A and 5, gave 50 per cent infectivity end points in chick embryos at $10^{-11.1}$ and $10^{-11.4}$ gm. of nitrogen, respectively, and in mice at $10^{-8.3}$ and 10^{-10} gm. of nitrogen, respectively.

As shown in Table I, the highest CCA activity obtained for a purified preparation of PR8 mouse lung virus was about 30,000 CCA units per mg. of nitrogen. On the other hand, purified preparations of PR8 virus obtained from allantoic fluid by comparable techniques always possess CCA activities in the neighborhood of 40,000 units per mg. of nitrogen. The latter titer is not increased by repeated adsorption on and elution from red cells. In order to ascertain whether the significantly lower titer of the mouse lung material was characteristic or was due to residual inert material, one of the preparations was subjected to a third adsorption on and elution from red cells. This process failed to increase the activity of the preparation and therefore indicates that virtually all extraneous inert material removable by this process has been eliminated when such preparations reach a CCA titer of about 30,000 units per mg. of nitrogen.

[2] It should be noted that, although preparations of virus obtained by a combination of the methods of adsorption on and elution from red cells and differential centrifugation possess higher CCA activities than any other preparations obtained in this laboratory they are often significantly less infectious than the preparations obtained by means of differential centrifugation alone. This is in accordance with the observation of Hirst and of many others that the infectious quality of influenza viruses is much more readily lost than is the red cell-agglutinating capacity. In the present instance, the loss of infectivity is likely due at least in part to the step in the procedure in which the virus-red cell mixture is subjected to a temperature of 37° C. for 1 to 2 hours, although this problem has not been thoroughly studied.

Size and Shape—Sedimentation data were obtained by Mr H K. Schachman and Mr J Hambleton by means of a Bauer-Pickels type ultracentrifuge (29, 30) equipped with a Svensson-Philpot optical system (31, 32) The sedimentation runs were made on preparation 1 of the mouse lung virus and on a preparation of allantoic fluid virus obtained under identical conditions Both preparations were examined in 0 1 M phosphate buffer at pH 7 The mouse lung preparation was at a concentration of about 3 mg per ml and that from allantoic fluid at 5 mg per ml As shown in Figs 1 and 2, a single boundary was observed in each case Sedimentation constants of 650 S and 602 S were calculated for the purified mouse lung and the purified allantoic fluid preparations, respectively If the value for the mouse lung preparation is corrected, as described by Lauffer and Stanley (12), for an observed relative viscosity of 1 05, it becomes 685 S

Electron micrographs of preparation 1 were made through the courtesy of the RCA Laboratories at Princeton with the kind assistance of Dr James Hillier An RCA type B electron microscope was used The material was applied to the collodion mounts at a concentration of 10^{-4} gm. per ml. in 0 1 M phosphate buffer, was allowed to dry, and then was washed with a small amount of distilled water and allowed to dry again Fig 3 shows a section of a field that was micrographed and enlarged to a total magnification of 13,500 It can be seen that the particles are circular in shape and measurements indicate that the average diameter is about 100 mμ

It is apparent from the electron micrographs as well as from the sedimentation data given above that the size and shape of the particles in highly purified preparations of mouse lung PR8 virus do not differ significantly from those of preparations from allantoic fluid (10, 12)

Chemical Tests and Analyses—Tests and analyses for key substances were made on some of the preparations listed in Table I

The preparations were essentially freed from phosphate buffer by 2 cycles of centrifugation from distilled water Most of the moisture was then removed by drying from the frozen state and by further drying the white, fluffy material thus obtained *in vacuo* over P_2O_5 at 60–80° *Many of the analyses* were made on aliquots of the solutions obtained by dissolving the dried material in 0 02 N sodium hydroxide. Nitrogen was determined by the direct nesslerization micro-Kjeldahl method of Koch and McMeekin (33) as modified for use in this laboratory (34), phosphorus by the method of King (35), and carbohydrate by the procedure of Tillmans and Philippi (36) An estimate of the amount of lipid was made on preparation 5 by extraction of the dry material with 3 1 alcohol-ether and after evaporation of the alcohol-ether, by extraction of the fatty residue with petroleum ether Tests for desoxyribonucleic and ribonucleic acids were made on fat free material with the Dische and Bial reagents, respectively (37, 38)

The averages of the nitrogen and carbohydrate values obtained by analyses on preparations 1, 2, and 4 were 10 1 per cent of nitrogen and 6 2 per cent of carbohydrate expressed as glucose Duplicate phosphorus determinations

on preparation 2A yielded a value of 1 06 per cent of phosphorus. In 10.8 mg of preparation 5 there appeared to be 38 per cent of alcohol-ether-extractable and 33 per cent of petroleum ether-extractable material. Positive color tests for both ribonucleic and desoxyribonucleic acids were obtained.

Analyses were also made on 2 preparations of sedimentable material from perfused normal lungs which were obtained after 3 to 4 cycles of differential centrifugation employing 15 minute periods of high speed centrifugation The 2 preparations were found to contain an average of 6 9 per cent of nitrogen, 1 4 per cent of phosphorus, and 3.5 per cent of carbohydrate The nitrogen and carbohydrate values are definitely lower and the phosphorus somewhat higher than those obtained for the virus preparations.

The analytical values given above and some of the physical properties of the mouse lung materials are compared in Table II with the corresponding properties of normal allantoic protein (10) and of highly purified preparations of PR8 influenza virus obtained from allantoic fluid

Isoelectric Point —

Measurements were made by Miss Mary Lloyd of the electrophoretic mobilities of several preparations of the virus and of the particles obtained by subjecting suspensions of perfused normal lungs to several cycles of differential centrifugation. The Northrop-Kunitz microelectrophoresis cell was employed under the conditions previously used in the study of virus preparations obtained from allantoic fluid (13) The preparations were all examined at a concentration of about 10 mg. per ml. Preparations of normal lung material and likewise those of PR8 virus obtained by centrifugation alone, possessed isoelectric points ranging from pH 4 1 to pH 4.5 On the other hand, 2 red cell preparations of the virus (1 and 5) appeared to be isoelectric at pH 5 4 (39) and 5.35, respectively In this respect these preparations correspond to concurrent red cell preparations of the virus obtained from allantoic fluid and to other highly purified samples previously studied (13) Three other preparations tested by this method, however, appeared to possess isoelectric points ranging from pH 6.75 to pH 7.2. Also the curves of mobilities *versus* pH of preparations 1 and 5 showed some aberration in the neighborhood of pH 7 while those of the remaining preparations had unusual plateaus between pH 5.5 and 6 8. However none of the preparations showed a minimum solubility at about pH 7 and, with the exception of preparation 3 and those obtained by centrifugation alone, all appeared to be as soluble in phosphate buffer at pH 7 as the best preparations from allantoic fluid. When the solubility of one of the preparations with an apparent electrophoretic isoelectric point at pH 7 05 was tested in a series of buffers, it behaved like a highly purified virus preparation from allantoic fluid in that it showed a minimum solubility in the neighborhood of pH 5 but differed slightly from the latter in that it was somewhat less soluble in the vicinity of pH 6.

The foregoing data may be summarized with the following conclusions. Mouse lung PR8 virus obtained by centrifugation alone has an isoelectric point near that of normal lung material Combined with other observations, this indicates that such preparations contain a large proportion of non-viral material Preparations of virus obtained by a combination of the methods of adsorption on and elution from red cells and centrifugation appear to possess two electrochemical natures, one of which tends toward an electrophoretic isoelectric

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The foregoing data may be summarized with the following conclusions. Mouse lung PR8 virus obtained by centrifugation alone has an isoelectric point near that of normal lung material. Combined with other observations, this indicates that such preparations contain a large proportion of non-viral material. Preparations of virus obtained by a combination of the methods of adsorption on and elution from red cells and centrifugation appear to possess two electrochemical natures, one of which tends toward an electrophoretic isoelectric

point at pH 5 4 and the other of which exerts an isoelectric influence in the region of pH 7 The former agrees with the solubility behavior of the material and coincides with the electrochemical behavior of the best preparations of

TABLE II

*Some Chemical and Physical Properties of Highly Purified Preparations of PR8 Influenza Virus and of Related Materials**

	Mouse lung PR8 virus	Allantoic fluid PR8 virus	Normal allantoic protein	Normal mouse lung particles
Nitrogen, *per cent*	10 1	10 2	9 0	6 9
Phosphorus, *per cent*	1 06	0 92	0 81	1 4
Carbohydrate (as glucose), *per cent*	6 2	6 5	7 7	3 5
Alcohol-ether–extractable, *per cent*	38‡	26 8	22 4	
Ribonucleic acid	Present	Present	Present	Present
Desoxyribonucleic acid	Present	Present	Present	Inconclusive
Sedimentation constant	*ca* 700 S	*ca* 700 S	*ca* 170 S	Inhomogeneous
Diameter of particles (from electron micrographs), *mμ*	*ca* 100	*ca* 100	*ca* 40	>40
Isoelectric point, *pH*	5 4	5 4	2 3	*ca* 4 2

* The data given for the mouse lung materials are those described in the text of the present report The nitrogen, phosphorus, and carbohydrate values for PR8 virus obtained from allantoic fluid represent the average from duplicate analyses on 3 lots of virus One of these lots was a pool of dried material from many preparations totaling over 2,400 mg All of the preparations in this lot and the preparation in another lot were obtained by a procedure of adsorption of the virus on and elution from chicken red cells similar to that used in the preparation of the mouse virus and described in the text The third lot was the homogeneous material obtained by centrifugal fractionation of partially purified virus (13) The values for the normal allantoic protein are the average of the values obtained with 5 different preparations procured by previously described methods (10) With the exception of the alcohol-ether–extractable figures, the differences between the analytical values presented for the virus derived from allantoic fluid and for the virus obtained from mouse lungs are no greater than those observed among individual preparations of virus from the same source It should also be noted that the nitrogen and phosphorus values for the allantoic fluid virus agree well with those reported from another laboratory (9)

‡ An approximate value since the single sample analyzed was too small for an accurate analysis

allantoic fluid virus The latter, however, appears to be characteristic of the mouse lung virus in that its influence was observed in all preparations thus far examined including one which had thrice been adsorbed on and eluted from red cells

Serological Tests—No serum has been prepared as yet against the highly

purified preparations of mouse lung virus. However, quantitative precipitin tests were made with other available sera with results which will be summarized here and described in detail elsewhere. Purified preparations of the mouse PR8 virus reacted strongly with antiserum to purified PR8 virus obtained from infectious allantoic fluid. Also this serum had a 50 per cent agglutination inhibition end-point of about 40,000 against 8 units of the mouse PR8 virus. The purified mouse PR8 virus failed to precipitate with antiserum to the sedimentable protein of normal allantoic fluid but did precipitate with anti serum to normal lung particles.

DISCUSSION

The most thorough comparison of virus preparations obtained from two different hosts was made by Loring and Stanley on tobacco mosaic virus isolated from tobacco and from tomato plants (2). In this instance, the proteins isolated from the two hosts were found to have the same chemical, physical, and biological properties. Much the same result has been obtained in the present study on influenza virus. The purified virus preparations isolated from mouse lungs and from allantoic fluid by identical procedures have been found to contain particles of the same size and shape, the same infectivity for chick embryos, essentially the same chemical composition, isoelectric point, and solubility, and common serological properties. Minor but characteristic differences have been observed in electrophoretic behavior and in precipitin tests with antisera to normal tissue particles. Also the lipid content of the mouse PR8 appears to be somewhat higher than that of the allantoic fluid virus although only one isolation of lipid was made and that, of necessity, from a rather small sample. In general, however, it is apparent that the properties of the virus preparations from mouse lungs and from allantoic fluid are remarkably similar and from this fact it can be concluded that the material in each instance probably represents influenza virus.

The author is indebted to Dr. W. M. Stanley for helpful suggestions during the course of this investigation and he was assisted by Mrs. Elmer Greey and Miss Mary Lloyd in perfusing and harvesting the lungs from the several thousand mice employed in the experiments.

SUMMARY

Highly purified preparations of PR8 influenza virus were obtained from perfused, infected mouse lungs by a combination of methods involving adsorption of the virus on and elution from chicken red cells and differential centrifugation. Such preparations were found to possess 50 per cent infectivity end points at 10^{-11} to $10^{-11.8}$, and 10^{-12} to $10^{-12.2}$ gm. of nitrogen in mice and in chick embryos, respectively. A sedimentation constant of 683 S was obtained for the material and electron micrographs revealed essentially spherical particles about 100 mμ in diameter. The material was isoelectric at pH 5.4.

and chemical analyses on several of the preparations indicated the presence of 10 1 per cent of nitrogen, 1 06 per cent of phosphorus, 6 2 per cent of carbohydrate and about 33 per cent of lipid Positive tests were obtained for both ribonucleic and desoxyribonucleic acids The virus was precipitated strongly by antiserum to purified PR8 virus obtained from the allantoic fluid of infected chick embryos and this serum inhibited the agglutination of red cells by the mouse virus to a dilution of about 40,000

In general, the properties of the virus isolated from infected mouse lungs were found to coincide with those of the virus obtained from the allantoic fluid of infected chick embryos

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EXPLANATION OF PLATE 1

FIG 1 A photograph of the Svensson schlieren diagram obtained during sedimentation of purified mouse lung PR8 influenza virus, preparation 1 Sedimentation was in 0 1 M phosphate buffer at pH 7 at 11,100 R.P.M. and at a concentration of about 3 mg per ml The photograph is the fourth in a series taken at 5 minute intervals

FIG 2 The same as Fig 1 except that the preparation was purified allantoic fluid virus at a concentration of 5 mg per ml

FIG 3 An electron micrograph of a purified preparation of PR8 influenza virus obtained from infected mouse lungs by a combination of the methods of adsorption on and elution from chicken red cells and differential centrifugation × 13,500

(Knight Purified PR8 influenza virus)

STUDIES ON PNEUMONIA VIRUS OF MICE (PVM)

I. The Precision of Measurements in Vivo of the Virus and Antibodies against it*

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That normal mice may harbor in their lungs a latent virus, capable of inducing fatal pneumonia in its natural host, was indicated in a previous report (1) The finding that this virus can induce manifest pulmonary infection in animals, which, under ordinary circumstances, harbor it with impunity, raises problems of obvious importance to a fuller knowledge of acute respiratory disease Moreover it raises the possibility that latent infectious agents may play a significant rôle in infections of the respiratory tract

A number of viruses have been procured by serial passage of mouse lungs during the past few years. Most of these agents have been referred to as "mouse pneumonitis viruses, or simply as 'pneumonitis viruses, and are now thought to belong in the so called psittacosis-lymphogranuloma venereum group of viruses (2) In addition to the agents of this group mice may harbor a latent pneumotropic virus which to distinguish it, we have termed "pneumonia virus of mice' (1) For convenience this virus will be referred to in the present studies as PVM It should be emphasized that PVM is to be sharply discriminated from the so called mouse pneumonitis viruses and can be differentiated readily from these agents on the basis of the following characteristics (1) It does not form elementary bodies (2) It is strictly pneumotropic (3) It is not infectious for chick embryos (4) It induces the development of solid immunity against itself following intraperitoneal injection in susceptible species (5) It is readily neutralized by specific immune serum in high dilution and (6) it does not give complement fixation in the presence of such immune serum except, as will be shown in a subsequent paper (3) under very special conditions.

It is our belief that much may be learned concerning the factors responsible for acute respiratory disease in animals, as well as in man, through the intensive study of latent pneumotropic agents. For this reason we have carried out a

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per cent saline suspension of mouse lungs infected with PVM while normal cotton rats were immunized by the intranasal injection of 0.25 cc. of a 10^{-3} dilution of virus suspension. Serum was obtained from these animals approximately 3 weeks following injection. The specimens of human serum used in this study were obtained from patients admitted to the Rockefeller Hospital. All sera were stored without preservative at 4°C.

Neutralization Tests.—The technique used in neutralization tests was identical with that employed previously in similar studies on influenza A virus (5). Serial dilutions in steps of 0.5 log units of virus suspension were each tested against a number of serial fivefold serum dilutions. All sera were heated at 56° C. for 30 minutes before they were diluted. As a routine, each mixture of virus and serum contained 10 per cent normal horse serum which had been treated as described above, and mixtures were held for 30 to 60 minutes at 4° C. before mice were inoculated with them. Each mixture of virus and serum was inoculated intranasally into a group of six lightly anesthetized mice; each mouse was given 0.05 cc. Neutralization tests with each serum studied were repeated on at least three separate occasions. At least two different suspensions of PVM were purposely used in tests with each serum, and each neutralization test was controlled by a separate titration of virus done simultaneously in similar mice. The observation of mice, the examination of their lungs, and the duration of the observation period were identical to those described above for virus titrations. The method of calculation of serum dilution end points is described below.

Calculation of End Points.—Both the virus titration and the serum dilution end points were calculated by the 50 per cent end point method of Reed and Muench (6). It has been found that the so called 50 per cent maximum score end point, described previously (5) is considerably more reproducible with PVM, than either the 50 per cent mortality or the 50 per cent pulmonary lesion end point. It is of interest that Lauffer and Miller (7) recently showed that this weighted end point was also the most reproducible in their studies on influenza A virus. Throughout these studies all end points on virus titrations and neutralization tests refer to the 50 per cent maximum score value, which for convenience will be designated as M.S. 50. It is important to emphasize that this end point is influenced not only by the frequency with which fatal and non-fatal pneumonia is induced in mice, but also by the extent of the pulmonary consolidation which develops in each mouse.

EXPERIMENTAL

Titration of PVM in Mice

Before it was possible to evaluate accurately the significance of differences in virus titration end points with PVM following various procedures, it was necessary to determine the experimental error, *i.e.*, the expected variation, of such titrations with this virus.

During a period of more than 2 years a large number of titrations of PVM in mice were carried out by the method described above. Numerous different suspensions of mouse lungs infected with this agent were tested repeatedly usually at intervals of 1 week. Between tests the suspensions were stored at −70°C. As will be shown below suspensions of PVM stored under these conditions for as long as 78 days showed no significant alteration in titer. From four to ten separate virus titrations were carried out at different times on each of eleven different suspensions of PVM. The 50 per cent maximum score end point (M.S. 50) was calculated, as described above, for each titration, and the geometric mean end point for each suspension was determined from all of the titrations done with it.

The results of this series of titrations with PVM in mice are shown in Table I. Each of the suspensions was initially centrifuged at 1,500 R.P.M. for 10

or 15 minutes and suspensions 1 to 7 received no further centrifugation Suspensions 8 to 11, however, were each centrifuged additionally at either 8,000 or 10,000 R.P.M. for 10 or 15 minutes before titrations were carried out

It will be seen that there was considerable variation among the individual titration end points obtained with each suspension, but that there was more marked variation between the mean end points found for different suspensions It should be noted, however, that there was only slight variation in the mean values obtained with all of the suspensions at corresponding steps in the successive titrations Furthermore it will be noted that the mean end point found

TABLE I

Results of Multiple Titrations of PVM in Mice

PVM mouse lung suspension		M S 50 end point Log										Geometric mean
		Successive titrations										
No	Centrifugation R.P.M.	1	2	3	4	5	6	7	8	9	10	
1	1 500	−4 53	−5 00	−4 00	−4 20	−4 11	−4 61	−4 42	−4 16	—	—	−4 38
2	"	−3 84	−3 55	−4 00	−3 57	−3 93	−3 01	−3 65	—	—	—	−3 65
3	"	−3 30	−3 56	−3 15	−2 73	−3 25	—	—	—	—	—	−3 19
4	"	−3 49	−4 62	−4 39	−5 00	−4 26	−3 60	−4 13	−4 50	−3 58	−3 51	−4 11
5	"	−2 45	−3 05	−2 45	−3 03	−2 86	—	—	—	—	—	−2 77
6	'	−3 18	−2 93	−3 04	−2 33	−3 76	−3 58	—	—	—	—	−3 13
7	"	−3 73	−4 19	−4 52	−4 65	−3 78	—	—	—	—	—	−4 17
1–7	(Geo Mean)	−3 50	−3 84	−3 65	−3 64	−3 71	−3 70	−4 07	−4 33	−3 58	−3 51	−3 72
8	8 000	−3 50	−3 62	−3 92	−2 63	−3 27	−2 91	—	—	—	—	−3 30
9	"	−2 93	−2 38	−2 48	−2 00	−2 28	−2 00	−2 48	—	—	—	−2 36
10	10 000	−3 58	−3 32	−3 35	−3 35	−3 22	—	—	—	—	—	−3 36
11	"	−3 96	−2 47	−3 50	−2 91	—	—	—	—	—	—	−3 21
8–11	(Geo mean)	−3 49	−2 95	−3 31	−2 72	−2 92	−2 45	−2 48	—	—	—	−3 00
Total	(Geo mean)	−3 50	−3 52	−3 52	−3 31	−3 47	−3 29	−3 67	−4 33	−3 58	−3 51	−3 49

for four of the seven suspensions which were centrifuged only at 1,500 R.P M. was higher than the corresponding end point determined for any of the suspensions centrifuged at either 8,000 or 10,000 R.P M. The mean end point for suspensions 1 to 7 was greater by log 0 72, or 5 2 times, than the corresponding end point for suspensions 8 to 11

Since the mean end points obtained with all of the suspensions at corresponding steps in the successive titrations (*i e* means found by vertical analysis of the data in Table I) were closely similar, whereas the mean end points obtained for each suspension (*i e* means found by horizontal analysis of the data in Table I) were widely dissimilar, it seemed probable that certain of the suspensions actually contained different concentrations of virus Moreover

it appeared likely that suspensions which had been centrifuged at either 8,000 or 10,000 R.P.M. usually contained less virus than those which had received only light centrifugation

In order to determine the significance of the differences in end points shown in Table I various computations were carried out with the available data. The deviation of each individual titration end point from the mean end point obtained for that particular suspension was found, and from this data the mean deviation of all the experimentally determined end points for each suspension was calculated. The variance, V of the end points obtained with each suspension was also calculated and from this value the standard deviation σ of the distribution of individual end points was computed It will be recalled, as has been pointed out elsewhere (7) that, for small sample statistics, the variance is defined as the sum of the squares of the individual deviations from the mean divided by one less than the number of end points, and that the variance thus defined is equal to the square of the standard deviation

TABLE II

Deviations Variances and Standard Deviations of Titrations of PVM in Mice

PVM mouse lung suspension		No. of titrations	M.S. 50 end point Log	Deviation From geometric mean Log			Variance	Standard deviation
No	Centrifugation R.P.M.		Geo mean	Least	Greatest	Mean	V	
1	1 500	8	−4 38	+0 04	+0 62	±0 26	0 108	0 329
2		7	−3 65	0 00	−0 64	±0 23	0 110	0 332
3	"	5	−3 19	−0 04	−0 46	±0 20	0 087	0 295
4	"	10	−4 11	+0 02	+0 89	±0 45	0 287	0 536
5	"	5	−2 77	+0 09	−0 32	±0 25	0 088	0 297
6	"	6	−3 13	+0 05	−0 80	±0 37	0 257	0 507
7	"	5	−4 17	+0 02	+0 48	±0 33	0 172	0 415
1–7	(Geo mean)	46	−3 72	±0 04	±0 60	±0 31	0 149	0 386
8	8 000	6	−3 30	−0 03	+0 62	±0 37	0 225	0 474
9	"	7	−2 36	−0 02	+0 57	±0 23	0 103	0 321
10	10 000	5	−3 36	−0 01	+0 22	+0 09	0 017	0 130
11	"	4	−3 21	+0 29	+0 75	±0 52	0 427	0 654
8–11	(Geo. mean)	22	−3 00	±0 08	±0 54	±0 29	0 147	0 384
Total	(Geo mean)	68	−3 49	±0 05	±0 58	±0 31	0 146	0 383

The extensive data accumulated in this series of computations were condensed as much as possible and only those which were essential to the determination of variation in titrations with PVM are presented in Table II. The least and greatest deviations of end points for individual titrations from the mean end point for all titrations on the same suspension as well as the

mean deviation found with each suspension are shown The variance and standard deviation of end points computed for each suspension are also given

It will be observed that the means of the least and greatest deviations for the entire series were log ± 0 05 and ± 0 58, respectively, and that the mean deviation of all the end points was log ± 0 31 It will also be noted that the standard deviation of the distribution of the end points for the whole series was 0 383 log units It will be recalled that the standard deviation is, in fact, a measure of the reproducibility of end point determinations and consequently that this figure is essential before it is possible to determine with accuracy the significance of differences between end points It can be shown that with any two individual end points determined on a single virus suspension, a difference of $2 \times \sqrt{2} \times$ the standard deviation, should occur only once in 20 times Therefore, in titrations with PVM, a difference between two individual end points on different suspensions of $2 \times 1\,415 \times 0\,383 = 1\,084$ log units or more, can be taken to indicate that the chances are at least 19 out of 20 that the virus concentrations of the two suspensions were not identical A difference of 1 084 log units obviously corresponds to a twelvefold difference between titration end points

From the foregoing considerations it can be shown that there is a very high probability that the differences between a number of the mean end points shown in Table I were significant, and indicate that certain of the suspensions studied did in fact contain different concentrations of virus Thus, the probability is at least 0 997 that suspensions 1, 4, and 7 contained a higher concentration of virus than any of the other suspensions, excepting only suspension 2, and that suspension 9 contained a lower concentration of virus than any other, excepting only suspension 5 Similarly the probability is at least 0 999 that the mean end point obtained with all the suspensions centrifuged at either 8,000 or 10,000 R.P M was significantly lower than that found with all the suspensions which were merely centrifuged lightly

Susceptibility of Mice Obtained from Different Breeders

One of the most important but least controllable factors which influences the reproducibility of virus titration end points is the susceptibility of the animal species in which tests are carried out If the degree of susceptibility remains relatively constant, the degree of variation encountered may not be great On the other hand, if for any reason susceptibility does not remain constant, enormous degrees of variation may result

It was shown previously (1) that titrations of PVM carried out simultaneously in Swiss mice of similar age obtained from eight different breeders showed wide variations in end points It is now possible to show that some of the differences observed previously were highly significant and indicated that among the various stocks of mice tested there were in fact wide variations in susceptibility

During the course of the present study Swiss mice were obtained for a time from a commercial breeder in Maine Titrations of PVM were carried out by the technique described above in these mice, and the same virus suspensions were also tested simultaneously in the other mice used throughout this study Moreover attempts were made to carry out serial intranasal lung passages with PVM in the mice received from Maine, and also their serum was tested for the presence of neutralizing antibodies.

It was found that the mean virus titration end point in mice received from Maine was less than log -1.0 whereas the corresponding end point in mice obtained from the usual source was log -3.60 It is obvious from the computations given above that this difference in end points is highly significant Furthermore it was found that repeated attempts to carry out serial passages with PVM in the mice received from Maine were unsuccessful and in each instance the virus was lost. It appears evident from the results of these experiments that these particular mice were almost, if not entirely resistant to infection with PVM It seems probable that their resistance was a manifestation of active immunity to infection with the virus since numerous specimens of serum obtained from them showed high levels of neutralizing antibodies against PVM.

In the light of these experiments and those previously reported, (1) it may be concluded that Swiss mice obtained from different sources may possess different degrees of susceptibility to infection with PVM

Effect of Centrifugation on Virus Titer

The results of centrifugation and ultrafiltration experiments reported previously (1) indicated that in our preparations PVM had a particle diameter of approximately 100 to 150 millimicrons On this basis it would be expected that centrifugation of suspensions of the virus at speeds up to 10,000 R.P.M. for periods of 10 to 15 minutes should not cause a significant reduction in titer

None the less it has been repeatedly observed in this laboratory that such centrifugation did, in fact, decrease the titer of suspensions of PVM. The difference in the end points obtained with suspensions which had been lightly centrifuged, *i. e.* 1,500 R.P.M. for 10 to 15 minutes, and other suspensions which had been centrifuged at either 8,000 or 10,000 R.P.M. for 10 to 15 minutes is shown in Table I Evidence was presented above which indicated that the fivefold decrease in titer observed following centrifugation at the latter speeds had a high probability of being significant.

Direct tests on the effect of centrifugation at either 8,000 or 10 000 R.P.M. upon titration end points were carried with four separate suspensions of PVM which had received the usual light initial centrifugation Titrations were performed as described above both before and immediately after centrifugation at the higher speeds The results of these tests are shown in Table III It will be seen that the end point obtained with each of the suspensions was lower following centrifugation than before it The mean decrease in the titer with all

the suspensions was log −0 93, which represents a reduction in the end point of 8 5 times The probability is at least 0 999 that this decrease in titer is significant, and consequently it can be concluded that approximately 80 per cent of the virus was sedimented under the influence of the relatively low gravitational fields applied in these experiments These results might be considered as evidence that the original estimates of the particle diameter of PVM were in error and that the virus is considerably larger than was thought previously In another communication (3) evidence will be presented which indicates that PVM is at least as small as was originally thought and may in fact be even smaller

TABLE III

Results of Titrations of PVM before and after High Speed Centrifugation

PVM mouse lung suspension	Centrifugation		M S 50 end point Log		Change in M.S 50 end point following centrifugation
	R.P.M	Minutes	Before centrifugation	After centrifugation	Log
A	10,000	10	−5 14	−3 96	−1 18
B	8,000	15	−2 57	−1 89	−0 68
C	"	"	−2 57	−1 79	−0 78
D	"	"	−3 13	−2 05	−1 08
Geometric mean			−3 35	−2 42	−0 93

Effect of Storage on Titer of PVM

The results of previous experiments (1) suggested that PVM is a very labile virus and becomes inactivated rapidly when held at room temperature in either broth or saline suspensions It was found, however, that the presence of normal horse serum in suspensions served to stabilize the virus and reduced the rate of inactivation

Before attempting to ascertain the reproducibility of immune serum dilution end points in neutralization tests with PVM, it appeared of importance to determine as precisely as possible the rate of inactivation of the virus at various temperatures It seemed probable that this information would be useful in the development of a reliable technique for neutralization tests with this agent

Suspensions of mouse lungs infected with PVM were prepared as described above and to them sufficient normal horse serum was added to give a concentration of 10 per cent. These suspensions were kept for various periods at 22° to 28°C , 4°C , and −70°C respectively Titrations in mice were carried out as described above with each suspension before and immediately following the period of storage

The results of these titrations are shown in Table IV With four suspensions which were kept for 1½ to 6 hours at either 22°C or 28°C the mean reduction

in end point following storage was only log −0.37 It will be noted, however, that with the suspension which was held for 24 hours at 28°C a decrease in titer of 1000-fold or more occurred With six suspensions which were held at 4°C

TABLE IV

Results of Titrations of PVM before and after Storage at Various Temperatures

PVM mouse lung suspension	Storage		M.S 50 end point Log		Change in M.S. 50 end point following storage
	Temp.	Time	Before storage	After storage	Log
	°C				
a	22	1½ hrs.	−2 93	−2 33	−0 60
b	"	2	−2 38	−2 18	−0 20
c		2½	−2 97	−2 40	−0 57
d	28	3	−3 83	−3 75	−0 08
"	"	6	"	−3 45	−0 38
"	"	24	"	<−0 75	>−3 08
Geometric mean*			*−3 19*	*−2 82*	*−0 37*
d	4	3 hrs.	−3 83	−4 00	+0 17
"	"	6	"	−3 77	−0 06
"	"	24	"	−3 59	−0 24
e‡	"	"	−2 28	−2 00	−0 28
"	"	48	"	−2 48	+0 20
f	"	"	−2 57	−1 61	−0 96
g‡	"	"	−1 79	−1 41	−0 38
h		"	−2 57	−3 05	+0 48
i‡	"		−1 89	−1 75	−0 14
Geometric mean			−2 76	−2 63	−0 13
j	−70	36 days	−3 32	−3 22	−0 10
k	"	48	−2 45	−2 86	+0 41
l	"	75	−3 50	−2 91	−0 59
m	"	78	−3 49	−3 51	+0 02
Geometric mean			−3 19	−3 12	−0 07

* Results obtained with suspension d held for 24 hours were not included.

‡ Suspension was centrifuged at 8,000 R.P.M. for 15 minutes before storage.

for from 3 to 48 hours the mean reduction in end point following storage was only log −0 13 Finally with four suspensions which were stored at −70°C. for periods of from 36 to 78 days, the mean change in end point was no greater than log −0.07

By use of the standard deviation, 0.383 log units, found for the distribution of

end points in titrations with PVM it can be shown that there was only one significant change in titer following storage in all of the tests carried out. The marked decrease in titer which was observed with the suspension held for 24 hours at 28°C is highly significant, and indicates that almost all the virus was inactivated during this period It can be concluded from the results of these experiments that suspensions of PVM prepared as described above and containing 10 per cent normal horse serum showed no significant decrease in virus titer following storage at 22° to 28°C, 4°C, and −70°C, respectively, for periods of at least 6 hours, 48 hours, and 78 days, respectively

In neutralization tests as carried out in this study, mixtures of serum dilutions and virus dilutions were held for periods no longer than 1 hour at 4°C and for additional periods no longer than 30 minutes at room temperature (22° to 28°C) It seems evident, in the light of the experiments just described, that during these intervals it would be exceedingly unlikely that any significant inactivation of virus occurred and consequently it appears possible to calculate, within the limits of expected variation, the amount of virus neutralized

Neutralization of PVM by Immune Serum

In a previous paper (1) it was shown that PVM is readily neutralized by specific immune serum as determined by the results of tests in mice with mixtures of the virus and serum Despite the clear qualitative significance of the results previously described, no attempt was made at that time to establish the quantitative limits of neutralization tests with this virus

In the intervening period experiments designed to yield information on the reproducibility of immune serum dilution end points in neutralization tests with PVM have been carried out and studies on the relationship between serum titer and the amount of virus used in such tests have been made

Neutralization tests were carried out as described above, with PVM and selected immune sera obtained from three species It is important to emphasize that both the serum dilutions and the virus dilutions were varied with respect to each other in each test in order that the whole range of the reaction might be studied. Each mixture of serum and virus was tested in a group of six mice. At least three separate neutralization tests under these conditions were performed with each serum so that the four or more end points found in one test could be compared with end points obtained in other tests on the same serum and the variations analyzed

The results of three separate neutralization tests with PVM and a pool of anti PVM cotton rat serum (C) are shown in Table V The results obtained in these tests were analogous to those obtained in similar experiments with rabbit and human immune serum, because they serve as typical examples they are presented in detail It will be observed that with approximately equal quantities of virus the serum dilution end points found in each of the three tests were similar, and that when log 1 4 or more M S 50 doses of virus were used, the ob-

served end points were almost identical in each test. It should be noted also, that whereas the decremental change in the quantity of virus used in these tests was log 0.5, the incremental change in the serum dilution end point had a mean value of only log 0.33 This indicates clearly that, under the conditions of these experiments, the relation between the serum dilution end point and the quantity of virus used was not one of simple multiple proportions Additional comments on this point will be given below

The results obtained in all of the neutralization tests carried out with a pool of immune cotton rat serum (C), an anti PVM rabbit serum (R), and an immune human serum (H), are presented graphically in Fig 1 The logarithm of the M.S 50 serum dilution end point obtained in each test was plotted against the logarithm of the number of M.S 50 doses of virus used It will be seen that the various serum dilution end points found with each of the three sera studied

TABLE V

Results of Multiple Neutralization Tests with PVM and Immune Cotton Rat Serum

Virus			Serum dilution end point M.S 50					Change in mean end point per step Log
Dilution Log	Approximate M.S. 50 doses Log	Change in amount of virus per step Log	Test No.			Geometric mean	Mean Deviation	
			1	2	3	Log	Log	
−1 3	2 4	—	1 269	1 185	1 238	−2 36	±0 06	—
−1 8	1 9	0 5	1.375	1 387	1 440	−2 59	±0 03	0 23
−2 3	1 4	"	1 1440	1 1360	1 1750	−3 18	±0 04	0 59
−2 8	0 9	"	1 1530	1 4750	1 2280	−3 41	±0 18	0 23
−3 3	0 4		1 6400	1 3910	—	−3 69	±0 10	0 28
Geometric mean	1 4	0 5				−3 0	±0 08	0 33

tended to fall along straight lines when the quantity of virus used in each test was taken into account and both variables were plotted as logarithms.

In previous communications (5 8) it was shown that there was a linear exponential relation ship between the immune serum dilution end point and the quantity of influenza A virus neutralized. It was found that under fixed experimental conditions the whole course of the neutralization of influenza A virus by specific immune serum could be expressed by the equation

$$y = bx^a$$

in which y was the quantity of virus neutralized b the intercept on the I axis, x the serum dilution end point, and a a constant. With either ferret, rabbit, or human immune serum and influenza A virus it was shown that this equation could be applied in each case the exponent a was found to have a value = 1 44 It is apparent that the slope of the straight line, which is a graphic expression of the linear relationship between the two variables, x and y is determined by the value of the exponent a

used for this diuresis test were fasted but allowed access to water for 18 hours prior to receiving a water load by stomach tube. The rate of urine excretion was followed at 30 minute intervals for a period of 180 minutes.

Urine sodium and potassium determinations were made by means of an internal standard flame photometer.

Blood pressures were determined according to the technique described by Kersten *et al.* (1947). The instrument used was a photoelectric tensometer model FAD-1 manufactured by Metro Industries.

RESULTS

A wide variety of tissues was found to possess systems capable of inactivating vasopressin during *in vitro* incubation. In the rat the greatest activity was found in homogenates of liver tissue (Table 1).

TABLE 1. INACTIVATION OF THE ANTIDIURETIC ACTION OF VASOPRESSIN (PITRESSIN) INCUBATED WITH HOMOGENATES OF TISSUES FROM RATS

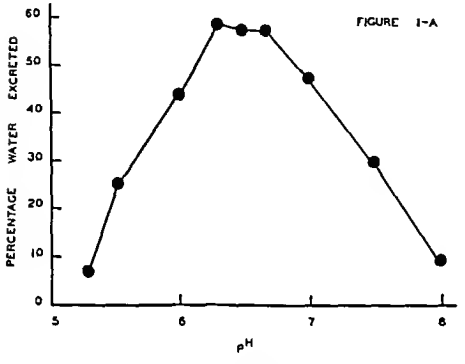
Tissue	Number animals	Percentage of administered water excreted at 90 minutes ±S.E.
Fresh liver	39	54 ±3.53
Heated liver	26	8 ±2.83
Spleen	12	21 ±3.65
Kidney	12	22 ±4.20
Whole blood	11	12 ±3.50
Skeletal muscle	12	16 ±2.21
Untreated controls	26	52 ±1.80

The vasopressin-inactivating system appears to have the properties of an enzyme. It is thermolabile, exposure to 80° C. for 10 minutes resulting in a complete loss of activity. The active principle is nondialyzable. When liver homogenates were dialyzed against ice-cold 1.0 per cent sodium chloride solution for 2 hours there was little or no loss of vasopressin inactivating ability. Dialysis for longer periods (4 or more hours) resulted in a loss of activity (Table 2). In all reactions tested the precipitation of proteins from the extracts was associated with a loss of vasopressin inactivating

TABLE 2. INACTIVATION OF THE ANTIDIURETIC ACTION OF VASOPRESSIN BY RAT LIVER HOMOGENATES WHICH HAD BEEN SUBJECTED TO VARIOUS EXPERIMENTAL PROCEDURES

	Number animals	Percentage of administered water excreted at 90 minutes
Untreated	10	51 ±3.60
Dialyzed 2 hours	7	47 ±4.20
Dialyzed 6 hours	8	33 ±3.67
Half saturation with ammonium sulphate	8	11 ±2.10
Addition of zinc sulphate (0.0005 M)	8	10 ±4.30
Addition of copper sulphate (0.0005 M)	8	8 ±3.52

ability. This loss of activity may be due to the fact that extracts left standing for longer than four hours show some decrease in activity. When the reaction of the homogenates was adjusted to pH 5.2, activity was almost completely destroyed. Further, half saturation with ammonium sulphate results in complete loss of activity (Table 2). The addition of zinc sulphate or copper sulphate (final concentration = 0.0005 M) to the incubation mixture resulted in a complete loss of inactivating ability. During incubation maximum activity was observed between pH 6.2 and 7.0



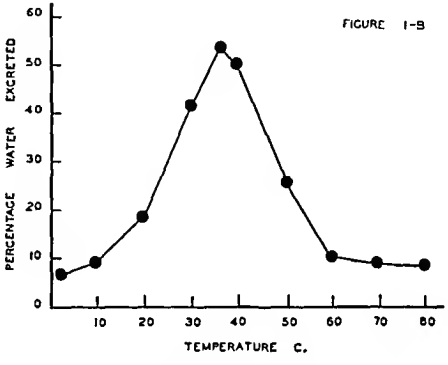


FIG. 1-A. Showing the effect on water excretion of test animals 90 minutes after injection of liver homogenates incubated at different pH levels. Each sample contained 20 milliunits of vasopressin per 0.1 gm. of tissue.

FIG. 1-B. Showing the effect on water excretion of test animals 90 minutes after injection of liver homogenates incubated for 30 minutes at different temperatures. Each sample contained 20 milliunits of vasopressin per 0.1 gm. of tissue.

(Fig. 1-A). The rate of inactivating of vasopressin increases sharply with a rise in temperature from 0° to 37° C.; however, with further increases in temperature there is a marked decline in activity (Fig. 1-B). Under optimal conditions 1.0 ml. of a 10 to 1 aqueous extract of rat liver tissue is capable of inactivating 100 milliunits of vasopressin during a 30 minute incubation period at 37° C.

No attempt has been made to determine the specificity of the vasopressin inactivating system nor to exclude the possibility that a nonspecific peptidase is involved.

It was observed that homogenates of rat liver tissues destroyed the pressor activity of vasopressin. Incubation mixtures containing homogenates of normal liver tissue failed to cause a rise in blood pressure of test rats while those made with heated liver extracts produced a marked increase in blood pressure (Table 3).

The inactivating system is not confined to tissues of the rat. Fresh human liver tissue contains a potent vasopressin inactivating system. It

TABLE 3. INACTIVATION OF THE PRESSOR ACTION OF VASOPRESSIN BY INCUBATION WITH RAT LIVER HOMOGENATES

	Number animals	Mean control blood pressure, mg. Hg	Mean blood pressure following injection, mm. Hg	Mean change in blood pressure, mm. Hg
Heated liver plus 20 mμ Pitressin	12	122	153	31
Fresh liver plus 20 mμ Pitressin	21	116	117	1
Saline plus 20 mμ Pitressin	15	122	142	20

was observed that the extracts of human liver (sample of tissue removed during abdominal surgery) not only destroyed the antidiuretic activity but also the natriuretic activity of vasopressin (Table 4).

TABLE 4. INACTIVATION OF ANTIDIURETIC AND NATRIURETIC ACTION OF PITRESSIN BY HUMAN LIVER

	Percentage water excreted at 90 minutes	Total sodium excreted at 90 minutes (meq. $\times 10^{-4}$)
Untreated controls	56.1	628
Fresh human liver (20 mμ Pitressin/0.02 g. tissue)	61.4	731
Heated human liver (20 mμ Pitressin/0.02 g. tissue)	8.4	1255

NOTE: All animals received one dose of water by stomach tube (4 ml. per 100 sq. cm. of body surface).

When extracts were made of liver taken from rats which had been given daily injections of 0.05 ml. of carbon tetrachloride in 0.05 ml. of paraffin oil for a period of 12 days, there was a sharp decline in the ability to inactivate vasopressin as compared with untreated controls (Table 5).

Extracts made of liver taken from rats which had been adrenalectomized for from 5 to 7 days showed a marked decrease in ability to inactivate the antidiuretic principle of vasopressin as compared with sham operated

TABLE 5. INACTIVATION OF THE ANTIDIURETIC ACTION OF VASOPRESSIN INCUBATED WITH RAT LIVER HOMOGENATES

Treatment of donor rats	Number animals	Percentage of administered water excreted at 90 minutes
		±S.E.
Untreated	26	54 ± 2.22
CCl_4 injected 12 days	12	39 ± 3.69
Adrenalectomized 5 days	21	41 ± 2.80
Adrenalectomized 9 days	15	31 ± 2.40
Dehydrated 24 hours	10	56 ± 3.51

controls. Liver obtained from animals in the terminal stages of adrenal insufficiency showed even less activity (Table 5).

DISCUSSION

The experiments reported here show that cell-free extracts of liver tissue contain an enzyme like system capable of inactivating the antidiuretic, pressor and natriuretic principles of posterior pituitary preparations (vasopressin). The deduction of its enzymatic nature is based upon thermolability, nondialyzability, protein nature, and the kinetics of its reactions. The specificity of the system has not yet been determined. It is possible that the enzyme is a peptidase but thus far it has not been identified.

The distribution of the inactivating system in various tissues confirms the observations of Heller and Urban (1935); Jones and Sclapp (1936); and Eversole, Birnie and Gaunt (1949) on the role of the liver in destroying the antidiuretic principle of posterior pituitary extracts.

The inactivating system is not confined to the tissues of the rat as the present study demonstrates its existence in human liver and previous investigations have found it to be present in the mouse (Birnie, Blackmore and Heller, 1952) and rabbit (Møller-Christensen, 1951).

Whether the system plays a physiological role in the destruction of endogenous posterior pituitary hormone has still not been definitely established. The observation that physiological alterations cause a change in the rate of inactivation suggests that the system may play a role in controlling water metabolism in the intact animal.

It has long been postulated that water retention observed following clinical and experimental liver damage is due, at least in part, to a decreased rate of inactivation of the antidiuretic hormone by damaged liver tissue (Ralli *et al.*, 1945; Labby and Hoagland, 1947). The observations reported here are consistent with such a hypothesis. That these observations do not explain entirely the water retention associated with liver damage is suggested by the observations of Shorr and Zweifach (1948) that the damaged liver itself may produce antidiuretic materials.

It is well established that adrenalectomized animals show a deficient diuretic response to administered water; this has been ascribed in part to an accumulation of antidiuretic material in the body fluids (Birnie *et al.*, 1949; Gaunt *et al.*, 1950). It has been postulated that his increase may be due to (a) the neurohypophysis being hyperactive following adrenalectomy, or (b) a decrease in the normal destruction of antidiuretic material by the tissues. The former possibility is open to serious question on theoretical grounds (Gaunt, 1951) and the present study provides evidence of a decreased destruction of antidiuretic material following adrenalectomy. While failure of the liver to inactivate antidiuretic material

would not explain all of the alterations in water metabolism associated with adrenal insufficiency, it is possible a contributing factor to the syndrome.

SUMMARY

1. Cell free extracts of rat liver tissue contain an enzyme-like system capable of inactivating the antidiuretic, pressor, and natriuretic principles of posterior pituitary preparations (vasopressin).

2. Extracts of liver tissue inactivated vasopressin more effectively than did extracts of spleen, kidney, skeletal muscle or whole blood.

3. Extracts made of liver taken from adrenalectomized rats showed a marked decrease in ability to inactivate the antidiuretic principle of vasopressin.

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THE ACCELERATING EFFECT OF ADRENALECTOMY ON REGROWTH OF HAIR IN THE THYROIDECTOMIZED RAT

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THE accelerating effect of adrenalectomy on hair growth in the rat is a well established fact, data on which have been reviewed by Baker (1951). This is a curious phenomenon for which there is no ready explanation. If hair growth is a part of general somatic growth, it would be expected to be altered with changes in the pituitary acidophils that secrete growth hormone (somatotrophic hormone, STH). A method for depleting the pituitary of acidophil granules is by thyroidectomy, after which there is known to be retardation of the hair growth. One might expect the loss of acidophil granules in the thyroidectomized rat to prevent the accelerated growth of hair after adrenalectomy.

This hypothesis was tested by the experiments to be reported here; the results, however, were found to be the direct opposite of those anticipated. This unexpected finding was clear-cut and constant.

METHODS

For the experiments were used groups of rats of a colony of Wistar albino strain, of the same sex, usually litter-mates, sometimes non-litter mates of approximately the same age, as follows:—(1) thyroidectomized rats with their adrenals intact, (2) adrenalectomized rats with their thyroids intact, (3) rats in which both thyroidectomy and adrenalectomy had been performed, (4) normal controls.

The rats that were thyroidectomized were operated on at 29 to 109 days of age. During the course of the experiments, evidence that thyroidectomy had been adequately performed was furnished by the retarded body-weight curve and the retarded regrowth of hair after shaving. On termination of the experiment, the characteristic changes in the pituitary (basophilic thyroidectomy cells containing intracellular hyaline material, and absence or marked diminution in granulated acidophils) was the final proof of thyroidectomy.

Bilateral adrenalectomy was carried out through the usual lumbar incision 34 to 222 days after thyroidectomy, thus allowing for the slow decay period of thyroid hormone in the circulation. From the experience of previous experiments (Zeckwer 1935), a long time interval was allowed for the slow development of cellular changes in the anterior pituitary following thyroidectomy. Adrenalectomized rats were given 1% NaCl

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drinking fluid. During the course of the experiments completeness of adrenalectomy in the rats with thyroids intact was attested by the much more rapid regrowth of hair after shaving, and at the termination of the experiment, proof of complete removal of adrenal tissue consisted of development of adrenal insufficiency on withdrawal of salt solution. At suitable time intervals the hair was shaved by electric clippers from the back and sides of all rats of a group, and the regrowth recorded by drawings and photographs. The same rats were shaved repeatedly, so that data could be obtained at varying time intervals before and after adrenalectomy. Periods up to 47 days after adrenalectomy were studied.

The rats were fed Purina dog chow and were kept in cages with solid sides and floors, open only at the top. Consequently the shaved rats were not exposed to draughts.

For histological studies, sections were prepared from the skin after fixation in formalin and stained with hematoxylin and eosin. The pituitaries were fixed in Helly's solution and stained with Mallory's acid fuchsin-aniline blue, orange G method.

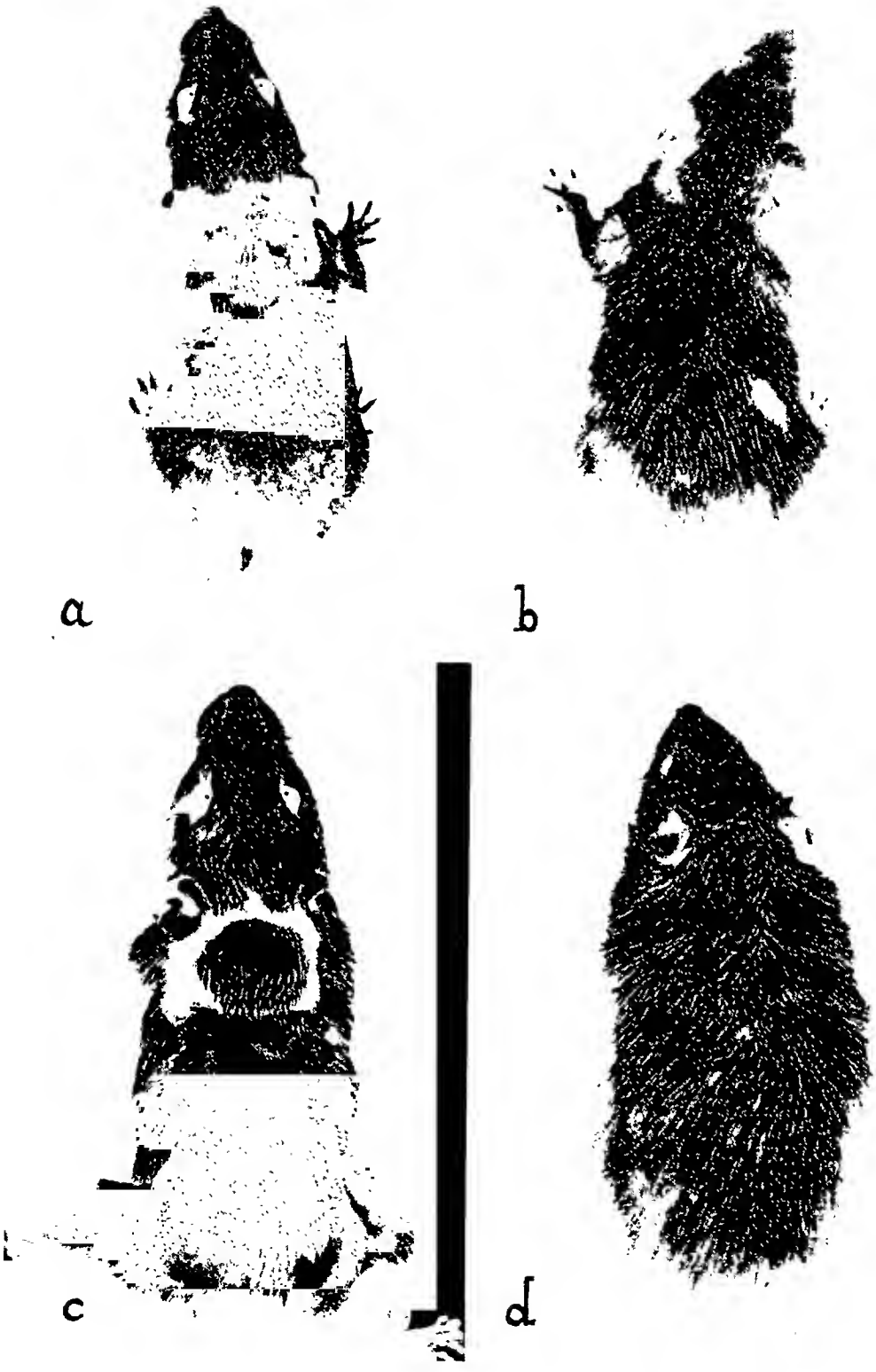
It was necessary to exclude from the analysis of the results: (a) a few very dwarfed rats thyroidectomized for a long period that survived adrenalectomy only a short time, (b) some thyroidectomized rats that had abnormalities of tooth formation which prevented adequate mastication and caused malnutrition, (c) a few adrenalectomized rats that did not develop adrenal insufficiency on withdrawal of salt. When a rat had to be excluded the remaining rats were compared with other groups of rats. In addition, sometimes rats served as their own controls when growth of hair in rats (with and without thyroid glands) was recorded before and after adrenalectomy. There remained for comparison 20 thyroidectomized rats with adrenals intact, 26 adrenalectomized rats with thyroids intact, 25 rats with combined thyroidectomy and adrenalectomy and 23 normal controls.

RESULTS

In normal control rats, regrowth of hair after shaving occurred in regular patterns and waves which have been recorded by various investigators, and details of which have been reviewed by Butcher (1934, 1951), Haddow (1945), and Wolbach (1951). This regrowth occurs symmetrically along a longitudinal axis and leaves certain areas of skin bare until the wave of growth approaches them. The location of the bare areas depends upon the time in the cycle when shaving was done. These cycles succeed each other rapidly in young rats; in older rats they progress slowly.

After adrenalectomy in rats with thyroids intact, this regular pattern of regrowth is usually not perceptible. Instead, there is a rapid diffuse

FIG. 1. Regrowth of hair, 30 days after shaving, in male litter-mate rats (49B). (a) Rat at 142 days after thyroidectomy (operation at 43 days of age). Body weight 228 grams. It can be seen that there is almost no regrowth of hair. (b) Rat at 142 days after thyroidectomy (operation at 43 days of age) and 35 days after bilateral adrenalectomy. Body weight 233 grams. In contrast to the appearance shown in the preceding figure (a) thick diffuse regrowth of hair has taken place. (c) Intact rat, showing normal patterns of hair growth, alternating with symmetrical bare areas. Body weight 288 grams. (d) Rat at 35 days after adrenalectomy. The operation was done at the same time as in rat (b). Body weight 284 grams. Thick diffuse growth of hair has occurred.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growth that occurs almost simultaneously over the entire shaved area. This effect did not appear immediately after adrenalectomy but required from 12 to 23 days for development. Once this latent period was passed, diffuse regrowth occurred rapidly after each shaving in a few days' time.

In thyroidectomized rats with adrenals intact, after about 5 weeks following operation (when thyroidectomy changes in the cells of the pituitary are extensive), the regular waves of regrowth occurred in normal patterns, but at a greatly reduced rate, and in many severely dwarfed rats that were thyroidectomized at an early age, the shaved areas remained entirely bald for long periods.

The effect of adrenalectomy in such previously thyroidectomized rats was striking and invariably the same. Adrenalectomy caused a diffuse regrowth of hair, in marked contrast to the condition of the same rat before adrenalectomy and in marked contrast to the appearance of control thyroidectomized rats with adrenals intact. However, this diffuse regrowth was much slower in development than in the adrenalectomized rats with thyroids intact. The latent period was at least one week longer. Eventually, the fur was thick and heavy and long in all adrenalectomized rats whether the thyroid was absent or intact, even in the case of extremely dwarfed long-time thyroidectomized rats that were losing weight. Figures 1 and 2 illustrate the external appearance of 2 experiments on litter-mates.

Microscopic sections of the skin of the rats show changes corresponding to the gross appearance of the hair. In the thyroidectomized rat the hair follicles are small and inactive and occur only near the surface. The dermis is very thin, and adipose tissue occurs between the muscle and dermis. In the thyroidectomized rat after adrenalectomy the follicles have become large and active and have grown deeply, down to the muscle. The dermis has thickened and adipose tissue has disappeared. The section of skin of a normal control rat, taken through a patch of hair growth, shows large active follicles. The adrenalectomized rat with thyroid intact shows good growth of many follicles.

Growth curves of the group of rats that were illustrated in Figure 2, are shown in Figure 3. It is evident that the thyroidectomized rats showed retarded growth.

FIG. 2. Regrowth of hair, 26 days after shaving, in male litter-mate rats (49R). (a) Rat 154 days after thyroidectomy (operation at 29 days of age). Body weight 172 grams. It can be seen that there is almost no regrowth of hair. (b) Rat at 154 days after thyroidectomy (operation at 29 days of age) and 26 days after bilateral adrenalectomy. Body weight 158 grams. Thick diffuse regrowth of hair has taken place. (c) Intact rat, showing symmetrical patterns of regrowth, alternating with bare areas. Body weight 282 grams. (d) Rat at 26 days after bilateral adrenalectomy. This operation was done at the same time as in rat (b). Body weight 290 grams. Diffuse thick growth of hair has occurred.

In microscopic sections the pituitary of the thyroidectomized rat and the rat with combined thyroidectomy and adrenalectomy have identical changes; namely, granulated acidophil cells have disappeared entirely in both. Each pituitary is filled with large basophilic thyroidectomy cells, many of which contain intracellular hyaline material. These thyroidectomy cells are regarded as cells producing TSH which is secreted in excess when the pituitary is released from the inhibiting effect of thyroxin.

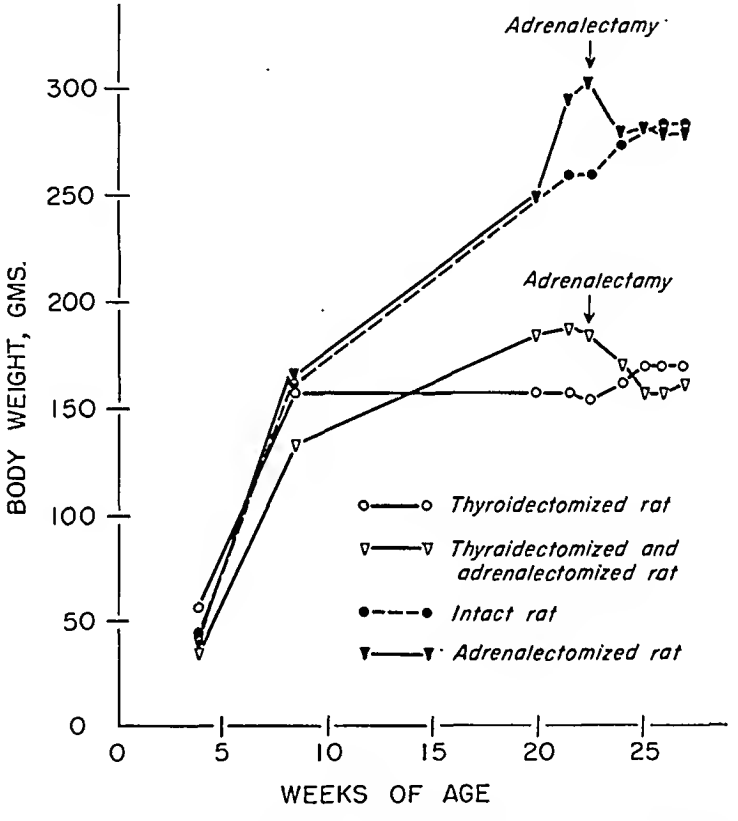

FIG. 3. Growth curve of litter-mate rats (49R) that are illustrated in Figures 2 and 3.

DISCUSSION

It is of interest to attempt a correlation of the structural with the functional changes in the pituitary. Several questions arise: Is the disappearance of granulated acidophils in the pituitary of the thyroidectomized animals a manifestation of decreased secretion of STH ? Does this mean that STH secretion is influenced by thyroid activity? Are the numerous acidophils in the pituitary of the adrenalectomized rats responsible for accelerated hair growth? Unfortunately, a simple explanation that body growth and hair growth are both manifestations of STH and are both correlated with numbers of acidophils does not hold because we find that

accelerated hair growth occurs in rats with combined thyroidectomy and adrenalectomy in which no granulated acidophils are present in the pituitary.

In spite of this apparent inconsistency, there is evidence accumulating from biochemical studies that growth hormone secretion is altered after thyroidectomy and after adrenalectomy.

That there may be an increased amount of growth hormone in adrenalectomized animals is indicated by the experiments of Engel (1951b) who states that in the adrenalectomized animal there is an increased rate of protein synthesis and a decreased rate of protein breakdown and thus presumably the adrenalectomized animal should show a greater growth rate than normal, if it were not obscured by reduced appetite of the animal. Analyses of the carcasses of normal rats restricted to the same food intake as adrenalectomized animals suggested chemical growth in the latter. He cites a thesis by J. Winternitz (1942) indicating that specific tissues may show enhanced growth rates after adrenalectomy.

Tepperman, Engel, and Long (1943) also cite Winternitz' experiments (1942) as suggesting that the removal of the adrenals which normally promote protein catabolism results in a relative hyperactivity of the anterior pituitary in promoting anabolism.

Wyman and Tum Sudan (1943) found increased growth of the epiphyseal cartilage of adrenalectomized rats, interpreted to mean that adrenalectomy releases growth hormone from the pituitary. It is of interest that they found that this increased growth of cartilage began near the end of the 2nd week after adrenalectomy, i.e. exactly at the time that elapsed before hair growth was accelerated in the present experiments.

Other biochemical studies seem to have a bearing on the question of whether growth hormone production is altered after thyroidectomy and after adrenalectomy. Hoberman (1950) in studies of amino acid and protein metabolism gives data which suggest the possibility that the secretion or synthesis of growth hormone is influenced by the activity of the thyroid gland, and he suggests that "in hypothyroidism the rapidity of breakdown of amino acids is due to a release of the catabolizing system from the inhibiting influence of growth hormone." He states that "adrenalectomy appears to be responsible for stimulation of protein synthesizing reactions whether performed on normal or hypothyroid rats" and he finds that "the nitrogen output is significantly lower in the absence of both adrenal and thyroid glands than after extirpation of either gland alone."

Is hair growth an expression of growth hormone (STH) activity? Hypophysectomy causes great retardation in regrowth of hair (Snow & Whitehead, 1935). We should not expect hair growth to be completely abolished by hypophysectomy, because, as Engel (1951a) suggests, it is generally

recognized that "hormones do not initiate or abolish any specific metabolic reaction, but simply modify the rate of existing ones." Both hair growth and body growth are suppressed by administration of ACTH or cortisone to the normal animal, so one would expect the growth of both would be increased after adrenalectomy, if the effects of growth were not masked by decreased food intake.

Van Dyke *et al.* (1950) suggest that "production of growth hormone by the pituitary is not under the control of the blood level of growth hormone." The evidence summarized in the present report suggests the possibility that the level of adrenal cortical hormone in the blood may be a means of controlling the rate of growth hormone secretion. Thus removal of the adrenals would remove an inhibition to the pituitary and release growth hormone secretion.

In the homeostatic regulation of carbohydrate metabolism, it is well established that the hypoglycemic effect of insulin secretion by the pancreas is counterbalanced by the 2 opposing factors of (1) anterior pituitary diabetogenic factor and (2) the adrenal cortical factor promoting gluconeogenesis. If the adrenal cortical factor is removed by adrenalectomy, one might anticipate that the anterior pituitary might compensate by secreting extra diabetogenic factor. Consistent with this idea is the fact that adrenalectomized rats, if given sufficient NaCl, will tend to maintain adequate levels of blood sugar. Since the anterior pituitary diabetogenic factor has been attributed to growth hormone STH, one might postulate that after adrenalectomy there might be compensatory increased secretion of growth hormone.

We should not expect accelerated hair growth to be demonstrable in such animals as dogs and cats in which fatal adrenal insufficiency occurs too rapidly.

Aside from these theoretical considerations, the observation of hair regrowth after shaving offers a very simple method for estimating the adequacy of thyroidectomy or adrenalectomy during the course of long time experiments, and may also give an estimate of rate of STH secretion even when body weight can not be taken as an index, due to inadequate food intake.

The lack of correlation between granulated acidophils and apparent STH secretion can not be explained at present. The absence of acidophil granules does not necessarily mean that no STH secretion is produced. The actively secreting cell discharges granules and then the cell reverts to the chromophobe appearance. Lack of granules may mean that there has been an accelerated rate of secretion by the acidophils to the point of exhaustion, where all hormone is discharged into the circulation and none is stored in the cell. Secretion rate and storage need not run parallel.

Whatever is the cause of the accelerated hair growth after adrenalectomy it is obvious that the changed metabolism takes several weeks to develop. This change, once established, is maintained for long periods. In the present experiment, no point was reached at which the acceleration of growth of hair disappeared.

Ralli and Graef (1945) reported that DOCA prevented the stimulating effect of adrenalectomy on regrowth of hair. At present it does not seem possible to place this finding in relation to the results of the present experiments.

A study of hair growth after combined thyroidectomy and adrenalectomy is recorded by Butcher (1941) who devised experiments to find out if accelerated hair growth after adrenalectomy was accomplished by thyroid secretion. He used very young rats, underfed so as to reduce the rate of hair growth, and thyroidectomized at 36 days of age. In some rats adrenalectomy was done at the same time, in others adrenalectomy was done 7–10 days and 3 weeks after thyroidectomy. He concluded that thyroidectomy had no effects on hair growth after adrenalectomy. However, his experiments do not extend over a sufficiently long period to give time for adequate decay of thyroid hormone in the circulation, nor could advanced pituitary changes take place. Furthermore, the periods of hair growth he studied were only 8 or 9 days. In brief, in Butcher's experiments, conditions were very different from those in the present experiments.

SUMMARY

Regrowth of hair after shaving was studied in groups of rats of the same sex and age: (1) rats thyroidectomized at 29 to 109 days of age; (2) rats thyroidectomized at the same time as in the preceding group and also adrenalectomized 34 to 163 days after thyroidectomy; (3) rats adrenalectomized at the same time as in preceding group; (4) intact controls.

Confirming previous studies, it was found that thyroidectomy retarded regrowth of hair.

In adrenalectomized rats, with their thyroids intact, there was a latent period of 12 to 23 days, after which hair grew diffusely instead of in the normal cycles, and at an accelerated rate. After adrenalectomy in previously thyroidectomized rats, there was a longer latent period of at least one week more than in those with thyroids intact, followed by a diffuse regrowth of hair that eventually equalled the growth of hair in adrenalectomized rats with thyroids intact. Microscopic appearance of skin was consistent with the gross findings.

After thyroidectomy, pituitaries showed the usual disappearance of acidophils and development of basophilic "thyroidectomy" cells. Subsequent adrenalectomy did not reverse these histological changes; that is, acido-

philic cells did not reappear. Nevertheless, from the results of other investigators, it seems likely that adrenalectomy does cause the pituitary to form more growth hormone.

In view of the fact that STH is commonly ascribed to the acidophil cells of the pituitary, the absence of acidophils in rats with combined thyroidectomy and adrenalectomy, in which accelerated regrowth of hair occurred, is an apparent inconsistency for which there is no explanation at present.

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A RAPID METHOD FOR THE QUALITATIVE AND QUANTITATIVE ESTIMATION OF THE PHYSIOLOGICAL ACTIVITY OF THE ADRENAL CORTEX[1]

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IT IS apparent to investigators interested in the physiology, pharmacology, or physiological chemistry of the adrenal cortical hormones that a rapid method for the qualitative and quantitative analysis of the hormonal secretions of the adrenal cortex would be of considerable value. It is the purpose of this communication to describe such a procedure and to present preliminary data resulting from the application of these techniques.

EXPERIMENTAL

For this particular study, dogs were chosen for the experimental animal. The dogs were sacrificed by an intracardiac injection of a lethal dose of nembutal. The adrenal glands were quickly removed and placed in ice. After removing the connective tissue and fat, the tissues were blotted, weighed, and slices prepared with a Stadie slicer. An aliquot was reserved for nitrogen analysis. The remainder of the slices were incubated in a large Warburg flask containing 20 ml. of the animal's own serum along with 10 mg. of adrenal cortical stimulating hormone (Armour lot #128-105R, potency 1.6 times that of LA-1-A standard), 50,000 units of penicillin, and 0.1 gm. of streptomycin. The vessel was gassed with 95% O_2—5% CO_2; the bath temperature was 37.5° C. and the time of incubation was 20 hours.

Following incubation, the adrenal slices and incubating medium were homogenized and subjected to fractionation similar to that described by Tennent, Whitla, and Florey (1951). The aqueous homogenate was treated with 5 volumes of acetone and the precipitated proteins filtered off. After removal of the acetone by vacuum distillation, the residual aqueous phase was extracted 5 times with ethyl acetate and dried over sodium sulfate. The bath temperature for all distillations was 40° C. The ethyl acetate was

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DISCUSSION

It is clear that following the incubation of relatively small amounts of adrenal cortical tissue, significant quantities of cortical steroid hormones can be recovered by the partition and chromatographic techniques. It is quite evident that the incubating procedure is of importance in estimating the actual elaboration of steroid hormones. The largest quantity of the predominating steroids obtained by direct isolation employing similar chromatographic techniques from beef adrenal glands was 400 μg. per pound of Compounds B and A (Zaffaroni and Burton, 1951). In the present experiments, as much as 100 μg. of Compound F could be recovered after incubating 1 g. of adrenal tissue. In the studies of beef adrenal glands cited (Zaffaroni and Burton, 1951), Compound F was present in the gland in quantities considerably less than Compounds B and A. In the present experiments with dog adrenal slices, Compounds F and E were predominant. It is certainly possible that this may be due to the difference in the species of animals employed (cf. Pfiffner, 1942). The discrepancy between the quantity of hormone present in the gland and the actual physiological elaboration receives support from the experiments of Hechter *et al.* (1951) who found that the chief hormones elaborated by perfused beef adrenal glands were Compounds F and B.

It is hoped that these techniques will be useful in evaluating adrenal cortical function during various stressing conditions. Some experiments along these lines are in progress and will be reported later.

SUMMARY

A relatively rapid method for the qualitative and quantitative measurement of the steroid hormone production of adrenal cortical tissue has been described.

The following steroids have been identified and quantitative estimation of their elaboration determined: Compounds F, E, A, S, and B. Compound F is produced in the greatest quantity; Compound E somewhat less, and smaller amounts of A, S, and B were found.

It is apparent that the steroid hormones recovered after incubation differ greatly in a quantitative manner from those recovered by direct isolation from the adrenal cortex.

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A QUANTITATIVE METHOD OF DETERMINATION OF THE DIABETOGENIC ACTIVITY OF GROWTH HORMONE PREPARATIONS*

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A NEW, rapid and quantitative method of determination of the diabetogenic activity of growth hormone preparations has been developed, based on the effect of this hormone on obese-hyperglycemic mice. The metabolic characteristics of this new Mendelian recessive condition have been described in previous publications (Bleisch, Mayer and Dickie, 1952; Guggenheim and Mayer, 1952; Mayer, Russell, Bates and Dickie, 1952; Mayer, Russell, Bates, and Dickie, 1952).

Particularly relevant to the question discussed here are the characteristics of the hyperglycemia in the obese mice. This develops as a progressive condition, usually after the 12th and before the 18th week of life. The precise time of onset seems to be conditioned by the presence or absence of genes other than "obese" in the genetic composition of the obese mice. The hyperglycemia is sensitive to the amount and the nature of the diet. It is highly sensitive to growth hormone, being either precipitated (during the prediabetic diet) or very much exaggerated (during the diabetic or hyperglycemic phase) by a single injection of growth hormone. By contrast, the blood sugar of non-obese mice are unaffected even by prolonged, massive treatment. Decreased glucose phosphorylation rates have been implicated in the etiology of the hyperphagia, in the light of the glucostatic theory of regulation of food intake (Mayer, 1952; Mayer and Bates, 1952).

Recent results (Mayer, unpublished) indicate that the obese animals are characterized by hyperfunction of the alpha cells of the pancreas and overproduction of a pancreatic hormone other than insulin. Growth hormone appears to be the trophic hormone corresponding to this new pancreatic factor.

METHOD AND RESULTS

The property of single injections or of a few injections of growth hormone to increase drastically the blood glucose of obese-hyperglycemic animals is the basis of the proposed

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method. Although reliable results were obtained with single injections in diabetic animals, maximum precision and homogeneity was obtained when using obese mice still in the prediabetic period injected for 3 consecutive days with 3 equal doses and effecting the determination on the 4th day. The Folin Ferricyanide colorimetric method (Folin,

TABLE 1. EFFECT OF THREE CONSECUTIVE DAILY INJECTIONS OF GROWTH HORMONE ON THE BLOOD SUGAR OF YOUNG HEREDITARILY OBESE-HYPERGLYCEMIC MICE

Dose (mg. growth hormone)	none (saline)	0.1	0.2	0.5	1	2	4	10
Number of animals	10	8	8	7	5	4	4	4
Mean blood sugar (mg. per cent)	160	207	218	230	248	303	355	507
Standard deviation (mg. per cent)	±20	±12	±9	±6	±3	±4	±15	±32

1930, 1932) following the Somogyi method of precipitation (Somogyi, 1945) was used. The animals were 3 to 4 months old, weighed 40 to 50 g. and were fed Purina Laboratory Chow. Sex introduces no variable in the determination.

A standard curve (given in tabular form in Table 1) was established using a usual (Armour) preparation. Doses given are the daily dosage. The results were shown to be independent of the method of preparation by comparing the standard Armour hormone with a preparation made according to the directions of Raben and Westermeyer (1951, 1952) for the isolation of "non-diabetogenic" growth hormone. (Comparison was made

TABLE 2. EFFECT OF MODE OF PREPARATION AND OF ADDITION OF OTHER PITUITARY HORMONES ON THE BLOOD GLUCOSE RESPONSE OF YOUNG HEREDITARILY OBESE-HYPERGLYCEMIC MICE

Treatment	Number of animals	Mean blood glucose and standard deviation (mg. per cent)
None (saline)	10	160 ± 20
0.2 mg. standard Armour growth hormone	8	230 ± 6
0.2 mg. Raben et al. growth hormone	6	225 ± 27
0.2 mg. growth hormone + 0.2 mg. ACTH	8	220 ± 9.2
0.2 mg. growth hormone + 0.2 mg. thyrotropic hormone	6	235 ± 15

on the basis of equal nitrogen retaining activity or growth promotion in the hypophysectomized rat.) Possible errors in the method due to contamination by other pituitary hormones were studied by determining the effect of adding daily large supplements of ACTH and thyrotrophic hormone to the standard growth hormone preparation—ACTH (Armour) even in large amounts did not modify the results. Thyrotrophic hormone (Armour) increased blood sugar values only if doses of the same order of magnitude than growth hormone were used. The preparation used (Armour) did, however, show some growth activity. (Thyroxine, in doses up to 100 μg. per animal does not increase blood sugar levels (Mayer *et al.*, in press).) These results are summarized in Table 2.

DISCUSSION

This method is simple, quantitative and speedy. It has been repeatedly used in this laboratory in studies of purification of growth hormone. It may

be emphasized again that, while the experimental conditions described represent ideal circumstances for the carrying out of the determination, it is possible, whenever available material is limited, to carry out the determination using single doses. A standard curve should be established with single doses of the reference preparation. Sensitivity, though not reproducibility, is increased when more diabetic mice (older animals) are used and when a high glucose diet is fed to the animals.

Preliminary results, indicating many similarities between goldthioglucose obesity (Waxler and Brecher, 1950) and the hereditary obese hyperglycemic syndrome suggest that goldthioglucose-treated animals, more readily available, may be used in this bioassay.

SUMMARY

A new method for the determination of the diabetogenic activity of growth hormone preparations, making use of the trophic effect of growth hormone on the blood glucose of hereditary obese-hyperglycemic mice is presented. The method is rapid and can be used with hormones prepared by different methods, even in the presence of other pituitary contaminants.

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HORMONAL FACTORS INFLUENCING FAT DEPOSITION IN THE INTERSCAPULAR BROWN ADIPOSE TISSUE OF THE WHITE RAT[1]

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INTRODUCTION

IT HAS already been reported that prolonged exposure to cold leads to a hypertrophy of the interscapular brown adipose tissue in the rat (Pagé and Babineau, 1950). Since in such animals metabolism is necessarily enhanced, it was thought worthwhile to investigate the effects of hyperthyroidism on the brown fat of rats kept at ordinary room temperature. The role of adrenal hormones was also studied in view of the findings of Fawcett and Jones (1949) that the functional integrity of the adrenal cortex is essential for the normal complement of lipids in brown adipose tissue. It has also been reported that during stress, changes in the weight and appearance of this tissue parallel changes in the adrenals (Selye and Timiras, 1949; Lemonde and Timiras, 1951.)

MATERIAL AND METHODS

Young albino male rats of the Wistar strain were used. They were housed in individual cages and fed a purified ration supplemented with crystalline vitamins. This ration contained 18% protein in the form of casein and 4% fat (Mazola corn oil). Thyroid powder (VioBin), when used, was incorporated in the ration at the level of 0.05%. Hormones were administered subcutaneously in the following daily doses: thyroxine-sodium (BDH) 5 mg./kg. of body weight; cortisone acetate (Merck), 2.5 mg.; DCA (Ciba), 2.5 mg; and ACTH (Armour), 2 mg. Thyroxine-sodium was dissolved in dilute sodium hydroxide and 0.9% NaCl was used as a diluent for other hormones when indicated.

Bilateral adrenalectomy was performed in a single sitting through two cutaneous incisions under ether anesthesia. Adrenalectomized rats were given tap water containing 1% sodium chloride *ad libitum*. The animals were sacrificed by decapitation under light ether anesthesia and the interscapular brown adipose tissue was immediately dissected and weighed. It was dried to constant weight *in vacuo* at 48° C. The dry matter was later homogenized in a chloroform-methanol mixture and the fat was extracted therefrom at

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room temperature according to the method of Folch *et al.* (1951). The fresh weight of the perirenal fat (left side) was used as an index of fat depots, Pagé and Babineau (1951) having found the amount of fat in this tissue to be a reliable guide to total body fat.

Statistical treatment was limited to the determination of the standard error by the usual formula where it is equal to $\sqrt{\Sigma d^2/n\ (n-1)}$; $n(n-2)$ was used in groups numbering less than ten animals. The probability of identity between values was calculated from standard tables for Student's t.

RESULTS

Thyroid feeding

Thyroid feeding to young rats for a period of two weeks led to a considerable hypertrophy of the brown adipose tissue. This gain in the fresh

TABLE 1. EFFECT OF THYROID FEEDING ON THE BROWN ADIPOSE TISSUE OF INTACT AND OF ADRENALECTOMIZED RATS

Group	Experiment 1*					Experiment 2†	
	Intact rats			Adrenalectomized rats		Adrenalectomized rats	
	Controls (beginning of expt.)	Controls (end of expt.)	Thyroid fed	Controls (end of expt.)	Thyroid fed	Controls (end of expt.)	Thyroid fed
No. rats	10	10	10	11	10	6	7
Mean body wt., gms.							
Initial	145	145	145	145	147	236	226
Final	—	208	183	147	150	302	266
Perirenal fat, mg.	569±50	942±110	410±60	negligible	negligible	1191±300	667±300
P‡		.01	.01			.7	
Brown adipose tissue							
Fresh wt., mg.	167±14	284±20	407±21	163±12	197±19	350±32	554±69
P		.01	.01	(.9)§	.25	.02	
Water content, mg.	75±5	130±11	130±7	108±9	109±6	172±16	190±9
P		.01	.9	(.01)	.9	.3	
Fat content, mg.	60±8	113±15	234±20	24±5	56±15	124±17	313±26
P		.01	.01	(.01)	.05	.01	
Fat-free dry matter content, mg.	32±3	41±3	43±5	31±2	32±4	54±7	51±13
P		.05	.8	(.8)	.8	.8	

* Experimental period: 2 weeks.
† Experimental period: 36 days; rats thyroid fed during the last 29 days.
‡ P = Probability of identity between the two values shown above.
§ Probability of identity between untreated adrenalectomized rats and controls killed initially.

weight of the tissue could be entirely accounted for by the increase in fat content since no change occurred in the water or fat-free dry matter by comparison with control rats killed on the same day (Table 1, Experiment 1). It is also seen that fat deposition in the brown adipose tissue was accompanied by a reduction of depot fat as reflected in the size of the perirenal fat. On the other hand, body growth in untreated animals results in a significant increase in all three fractions of the brown fat. This is the general picture which was invariably reproduced in all experiments of this type.

The effect of adrenalectomy and of thyroid feeding to adrenalectomized rats was studied concurrently. In the case of the non-treated animals, the

size of the brown fat at the termination of the experimental period was the same as that of control rats killed initially. Its composition was significantly altered, however, the fat content dropping markedly and being replaced by an equal quantity of water (Table 1, Experiment 1). It might be argued that the stationary weight of the brown adipose tissue was consequent upon the lack of gain in body weight of these animals. However, a similar growth inhibition of this tissue was observed in a subsequent experiment where the adrenalectomized rats registered substantial gains in body weight (Table 2). The results seem to confirm fully the conclusion reached by Fawcett and Jones (1949) and mentioned above.

TABLE 2. EFFECT OF DCA ON THE BROWN ADIPOSE TISSUE OF INTACT AND ADRENALECTOMIZED RATS

Group	Intact rats			Adrenalectomized rats	
	Controls (beginning of expt.)	Controls (end of expt.)	DCA*	Controls (end of expt.)	DCA*
No. rats	10	10	10	12	12
Mean body wt., gms.					
Initial	140	141	140	140	140
Final	—	231	226	202	218
Perirenal fat, mg.	277 ± 35	1078 ± 156	1148 ± 159	389 ± 95	549 ± 60
P		0.01	.7	.2	
Brown adipose tissue:					
Fresh wt., mg.	248 ± 20	291 ± 21	291 ± 16	245 ± 18	244 ± 16
P		.2	.9	.9	
Water content, mg.	—	142	126	126	99
Fat content, mg.	—	101	120	74	100
Fat-free dry matter content, mg.	—	48	45	45	45

* Administered during the last two weeks of a three week experimental period.

Thyroid feeding to adrenalectomized rats caused a slight but not significant increase in the fresh weight of the brown fat (Table 1, Experiment 1). This increase was confined to the fat fraction, however, and on this basis it is significant at the 5% level. Identical results were later obtained in adrenalectomized rats receiving thyroxine subcutaneously (Table 3). In contrast to these findings, when larger rats were adrenalectomized and fed thyroid powder for an extended period of time, there resulted a hypertrophy of the brown fat (Table 1, Experiment 2) comparable in degree and nature to that observed in intact rats similarly treated. It is doubtful if one can exclude in such a prolonged treatment the possible intervention of accessory adrenal tissue or even the cortical-like action of androgens. These same rats were also reasonably well supplied with fat reserves in contrast to the previous ones so that in the smaller rats, limited response to thyroid

feeding or to thyroxine administration may have been partly due to lack of mobilizable fat.

Desoxycorticosterone acetate

Results shown in Table 2 indicate that the administration of DCA has no effect on the size of the brown adipose tissue either in intact or in adrenalectomized rats, nor does it affect significantly the amount of depot (perirenal) fat. Analyses of the brown fat were carried out on pooled samples so that the significance of any observed difference cannot be evaluated statistically. It may be noted, however, that in both treated groups there occurs a rise in the fat content of the brown adipose tissue and a concomitant decrease in water, a unique finding in all our studies to date.

Cortisone and thyroxine in adrenalectomized rats

The effect of cortisone in adrenalectomized rats receiving thyroxine or not was next investigated. Adrenalectomized controls received no treatment or were given thyroxine alone (Table 3). It is seen that cortisone alone caused a significant increase in the size and fat content of the brown adipose tissue as compared to the untreated controls. In fact, this hypertrophy is as large as that observed following thyroxine or cortisone treat-

TABLE 3. EFFECT OF THYROXINE, CORTISONE, AND OF THYROXINE + CORTISONE ON THE BROWN ADIPOSE TISSUE OF ADRENALECTOMIZED RATS

Group	Control	Thyroxine	Cortisone	Thyroxine + cortisone
No. rats	10	6	10	10
Mean body wt., gms.				
Initial	115	119	115	116
Final	135	125	132	115
Perirenal fat, mg.	88 ± 20	negligible	215 ± 30	112 ± 20
P vs controls			.01	.4
Brown adipose tissue				
Total fresh weight, mg.	254 ± 18	309 ± 25	403 ± 19	546 ± 42
P vs controls		.1	.01	.01
P vs cortisone group		.01		.01
Water content, mg.	152 ± 9	162 ± 6	169 ± 7	174 ± 10
P vs controls		.1	.1	.3
Fat content, mg.	62 ± 8	99 ± 17	182 ± 16	316 ± 33
P vs controls		.05	.01	.01
P vs cortisone group		.01		.01
Fat-free dry matter content, mg.	40 ± 5	48 ± 4	52 ± 6	56 ± 5
P vs controls		.1	.07	.02

Experimental period: 14 days; thyroxine or cortisone given the 9th, 10th and 11th day.

ment in intact rats (Tables 4 and 5). When given in conjunction with thyroxine, the increase in weight and fat content is double that obtained with cortisone alone.

These results would indicate that cortisone administration restores the action of thyroxine which is otherwise greatly impeded by adrenalectomy.

Cortisone and thyroxine in intact rats

In intact animals, the action of thyroxine is similar to that obtained by thyroid feeding (Tables 4 and 5). Cortisone has an effect comparable to

TABLE 4. EFFECT OF THYROXINE AND OF CORTISONE ON THE BROWN ADIPOSE TISSUE OF INTACT RATS

Group	Control	Thyroxine	Cortisone
No. rats	20	10	8
Mean body wt., gms.			
Initial	135	137	135
Final	170	149	140
Perirenal fat, mg.	436 ± 37	288 ± 37	328 ± 50
P vs controls		.02	.1
Adrenal wt., mg.	23.7 ± 0.6	26.6 ± 0.6	19.0 ± 1.5
P vs controls		.01	.01
Brown adipose tissue:			
Total fresh wt., mg.	326 ± 19	461 ± 26	426 ± 27
P vs controls		.01	.01
P vs thyroxine group			.4
Water content, mg.	156 ± 7	157 ± 9	177 ± 7
P vs controls		.9	.05
Fat content, mg.	124 ± 12	254 ± 17	190 ± 17
P vs controls		.01	.01
P vs thyroxine group			.02
Fat-free dry matter content, mg.	46 ± 3	50 ± 4	59 ± 6
P vs controls		.4	.08

Experimental period: 6 days; thyroxine or cortisone given on the 1st, 2nd and 3rd day.

that of thyroxine with this difference that the water content rises significantly and that the deposition of fat is less pronounced (Table 4). Seifter *et al.* (1951) have also reported an increase in the size and fat content of this tissue in pregnant rats given cortisone. On the other hand, Antopol (1950) working on mice and using relatively larger doses of cortisone observed an atrophy of the brown fat. Sala *et al.* (1951) report that this tissue regresses under the influence of cortisone administered over an extended period of time.

When cortisone is given in conjunction with thyroxine, an apparently

TABLE 5. EFFECT OF THYROXINE, THYROXINE+CORTISONE AND OF ACTH ON THE BROWN ADIPOSE TISSUE OF INTACT RATS

Group	Control	Thyroxine	Thyroxine + cortisone	ACTH
No. rats	12	12	12	12
Mean body wt., gms.				
Initial	134	134	134	134
Final	174	153	132	171
Perirenal fat, mg.	411 ± 50	90 ± 10	100 ± 10	421 ± 60
P vs controls		.01	.01	.9
Adrenal wt., mg.	23.7 ± 0.7	28.1 ± 1.2	18.6 ± 1.4	25.8 ± 0.7
P vs controls		.01	.01	.08
Brown adipose tissue				
Total fresh wt., mg.	310 ± 15	441 ± 15	544 ± 29	367 ± 22
P vs controls		.01	.01	.05
P vs thyroxine group			.01	
Water content, mg.	156 ± 4	151 ± 5	161 ± 9	174 ± 9
P vs controls		0.5	0.6	0.1
P vs thyroxine group			.3	
Fat content, mg.	107 ± 11	240 ± 9	333 ± 19	134 ± 14
P vs controls		.01	.01	.2
P vs thyroxine group			.01	
Fat-free dry matter content, mg.	47 ± 1	50 ± 3	50 ± 6	59 ± 4
P vs controls		.3	.6	.01

Experimental period: 8 days; Rats injected daily, the 6th day excepted.

additive response is obtained (Table 5). It is to be noted that cortisone induces an atrophy of the adrenals even in the presence of excess thyroxine. The fact that this hormone is equally potent whether the adrenals are removed or atrophied points to its preponderant role among cortical hormones with respect to fat deposition in the brown adipose tissue.

ACTH in intact rats

The action of ACTH is reported in Table 5. The dosage used was too low to increase significantly the size of the adrenals. Nevertheless, the brown adipose tissue was significantly hypertrophied and a general increase in all three fractions occurred, the rise in fat-free dry matter content being statistically significant. This response differs somewhat from that obtained with thyroxine or cortisone and is more reminiscent of spontaneous changes occurring during body growth (Table 1). Baker, Ingle and Li (1950) have observed a higher fat content of the brown adipose tissue in rats treated for 21 days with ACTH. From the trend shown in Table 5, it can be assumed that a longer treatment would have yielded similar results.

Hormonal treatment and depot fat

With respect to general fat stores, it can be seen that hyperthyroidism led to a considerable decrease in the amount of perirenal fat in all cases. Cortisone, on the other hand, seemed to prevent the loss of fat consecutive to adrenalectomy although it did not affect body weight. It also afforded some measure of protection against fat depletion in thyroid-treated adrenalectomized rats. These results are in accordance with the findings of Stoerk and Porter (1950). In intacts rats, neither cortisone nor ACTH had a perceptible effect on depot fat and the same seems to apply to DCA in intact and in adrenalectomized animals.

DISCUSSION AND CONCLUSIONS

Of chief interest are the findings: (1) that thyroxine elicits a minimum response in the absence of the adrenals; (2) that cortisone is equally effective in intact and in adrenalectomized rats; and (3) that an enhanced response is obtained when the two hormones are combined either in intact or adrenalectomized animals. There thus appears to exist a synergism between cortisone and thyroxine in respect to fat deposition in the brown adipose tissue. It may be that excess thyroxine given alone exhausts the cortisone producing capacity of the adrenal while cortisone given singly inhibits the production of thyroid hormone as some reports indicate (Money *et al.*, 1951; Lederer, 1952).

The origin of this extra fat in the brown adipose tissue remains to be determined. Experiments now underway seem to indicate that it is mobilized from fat stores in other regions. In view of the reported antilipogenic action of cortisone, one would hardly expect this fat to have been synthesized *in situ*. It is difficult to correlate our findings with those of Engel and Scott (1951) regarding the effect of cortisone on glycogen deposition in the brown fat. These authors express the glycogen concentration on a fat basis and should cortisone have stimulated fat deposition, it would follow that glycogen concentration would suffer an apparent decrease unless the whole tissue content in this substance rose proportionately.

Glycogen deposition in the brown adipose tissue is often considered as indicative of fat synthesis (Wertheimer and Shapiro, 1948). However, if the fat laid down in the brown adipose tissue originates elsewhere, one may ponder whether it is later oxidized directly or over the pathway of carbohydrate synthesis. Ingle (1950) has envisaged the possibility of fat conversion to carbohydrate under the influence of corticosteroids and Segaloff and Many (1951) suggest that extra urinary glucose in phlorizinized rats may have arisen from fat. ACTH and cortisone were among the compounds having a marked effect on gluconeogenesis and cortisone was much less

ketogenic than other such substances. Wells and Kendall (1940), in similar studies, had already reported a synergism between the actions of Compound E and of the thyroid gland so that the full exhibition of either was dependent upon the presence of adequate amounts of the other hormone. The similar interrelationship between cortisone and thyroxine in respect of gluconeogenesis and of fat deposition in the brown adipose tissue is striking although no link between the two processes has been shown to exist.

SUMMARY

1. Thyroxine and cortisone both promote the accumulation of fat in the brown adipose tissue of intact rats and their actions appear to be synergistic. Little or no change occurred in the water and fat-free dry matter contents.

2. In adrenalectomized animals, the response to thyroid hormone was minimal while the action of cortisone administered alone or in conjunction with thyroxine was comparable to that obtained in intact rats.

3. The administration of DCA caused no change in the size of the brown fat.

4. ACTH caused a slight hypertrophy of the brown adipose tissue, characterized by increases in all three fractions studied.

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A SODIUM-EXCRETING EFFECT OF DESOXYCORTICOSTERONE IN ADRENALECTOMIZED MICE*

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METHODS recently reported for the bioassay of desoxycortisosterone (DOC) (Dorfman, 1947; Dorfman, 1949; Deming and Luetscher, 1950; Spencer, 1950; Simpson and Tait, 1950, 1952: Luetscher and Demng, 1951; Marcus, Romanoff and Pincus, 1952) have utilized the action of DOC in adrenalectomized animals in decreasing urinary excretion of sodium, in increasing the urinary excretion of potassium or in effecting a combination of these actions. Little quantitative information has been published regarding the optimal sodium loading of the animals used for bioassay. Workers employing isotopic techniques have favored a low sodium load (Dorfman, 1947; Simpson and Tait, 1952) while those using the alteration in the urinary excretion of sodium as determined chemically or by flame photometer have favored a high salt load (Luetscher and Deming, 1951; Spencer, 1950; Marcus, Romanoff and Pincus, 1952). Although much is known concerning the effect of water loading in adrenalectomized animals (Gaunt, 1944, 1950; Hays and Mathieson, 1945; Birnie, Eversole and Gaunt, 1948; Frost and Talmage, 1951) few of these results can be applied to determining the optimal water load for the bioassay of salt retaining steroids, because urinary sodium excretion was not adequately measured.

The present studies were initiated to define more precisely the optimal sodium and water loads in adrenalectomized mice used for the bioassay of DOC. During the course of the sodium loading experiments, DOC was found to have a sodium excreting effect in small doses, and the usual sodium retaining effect in larger doses. Strain differences in sensitivity to DOC were noted in respect to both sodium excretion and sodium retention.

METHODS

Method 1

Male mice, strain CF1 Carworth Farms and strain C57 BBF1 Roscoe B. Jackson

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Memorial Laboratory, weighing from 20 to 25 grams were adrenalectomized using a single dorsal skin incision. These mice were maintained postoperatively on 0.9% sodium chloride solution, hereafter called saline, and "Friskies" dog biscuit containing 2.8 mg. sodium and 4.8 mg. potassium per gram of biscuit. Tests were performed from the fourth to the fourteenth post-operative day. Sixteen hours before assay solid food was removed and the animals were given a 10% solution of glucose or sucrose to drink. Desoxycorticosterone (free alcohol) dissolved in 10% ethanol was used throughout these studies. The control mice received 0.2 ml. 10% ethanol intraperitoneally, approximately 20 minutes before the assay was begun. The appropriate dose of sodium loading solution was calculated on the basis of body weight and injected subcutaneously using a number 25, two inch needle. In addition to sodium chloride the loading solution contained 5% glucose and four m.eq. per liter potassium. Urine was forcibly expressed by manual pressure over the bladder and the animals were placed in individual 600 ml. beakers with $\frac{1}{4}$ inch wire screen platforms over the bottom of the beakers. At the end of 5 hours the animals were removed and any feces present were discarded. Urine was again forcibly expressed from the bladder into the beakers. The contents of the beakers were transferred to volumetric flasks using four washes of distilled water. The total amounts of sodium and potassium in the urine were then determined with the Weichselbaum-Varney flame photometer.

Method 2

It was noted that the physical condition of the mice was often poor using Method 1 and the mortality during assay occasionally was 10–20%. For this reason the following modification was investigated. Nineteen hours before assay solid food was omitted and a solution of 10% glucose in 0.9% saline was used for drinking. Three hours before assay the mice were given only 10% glucose to drink. During the 5 hour assay period a cube of table sugar was placed in each beaker. Strain BBF1 mice ate the sugar very well, but strain CF1 mice largely ignored it. The sugar used contained approximately 0.06 mg. of sodium and 0.01 mg. of potassium per gram. In view of the small amount of sugar contaminating the urine, this source of error can be overlooked.

RESULTS

Strain CF1 Mice, Method 1

Mice of this strain were given loading solutions containing 0.5 mg., 0.3 mg., 0.1 mg. and 0.025 mg. of sodium chloride per gram of body weight administered subcutaneously in a volume of 0.5 ml. To observe the effect of the volume of loading solution on sodium excretion and DOC sensitivity the 0.5 mg. per gram, 0.1 mg. per gm. and 0.025 mg. per gram doses of sodium chloride were also injected in a volume of 2 ml. The excretion of sodium per gram of body weight under these experimental conditions with and without DOC are presented graphically in Figure 1. The decrease of urinary sodium produced by DOC was most evident with 0.5 mg. per gram of weight in a volume of one half ml. With this load a significant decrease in sodium excretion was apparent with 10 μg. of DOC ($P = 0.007$). When the same amount of sodium chloride was given in a volume of 2 ml. a dose of 25 μg. of DOC was required to establish a significant decrease in sodium

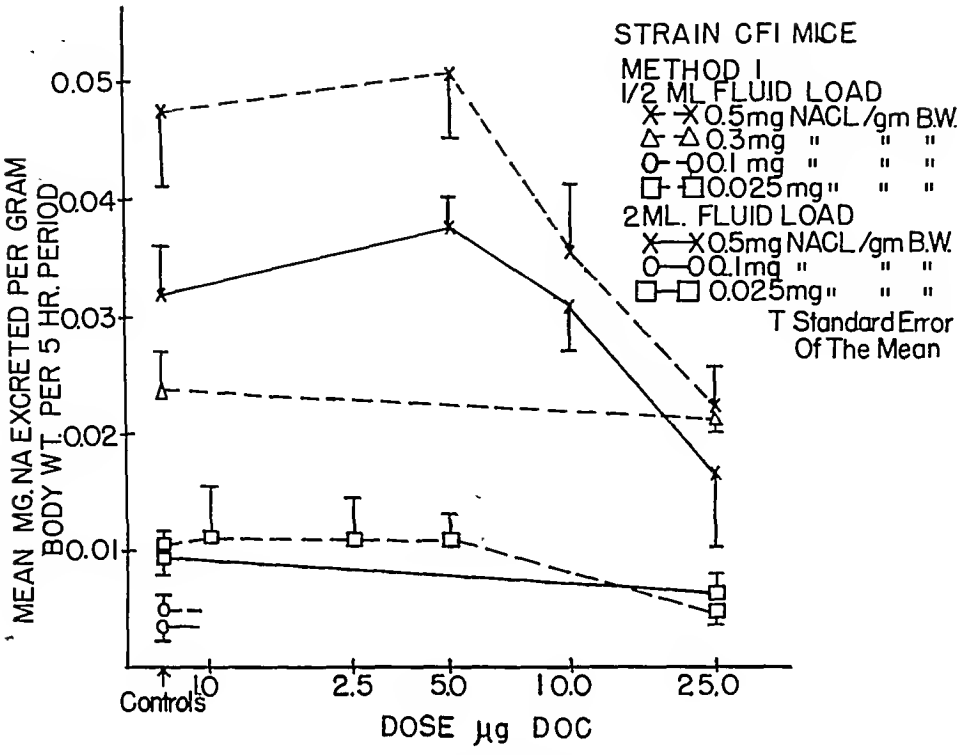

FIG. 1. The urinary sodium excretion in the strain CF1 mouse and its response to desoxycorticosterone with various fluid and NaCl loads. Standard error of the mean indicated in only one direction to avoid overlap. Each point represents the mean of from 8 to 35 animals.

excretion. With sodium loads less than 0.5 mg. of NaCl per gram of weight the control sodium excretion was correspondingly reduced. The absolute decrease in sodium excretion following DOC under these circumstances was small and too variable to be useful for assay. From these results pretreatment with 0.5 mg. NaCl per gram of body weight in 0.5 ml. was the most favorable condition for demonstrating the salt-retaining action of DOC.

Strain CF1 Mice, Method 2

Allowing adrenalectomized mice access to saline drinking water until 3 hours before assay in effect increases the salt load. The excretion of sodium with and without DOC under these conditions is presented graphically in Figure 2. Urinary sodium actually increases with doses of 5 and 10 µg. of DOC. Not until a dose of 25 µg. of DOC was given did a fall in urinary sodium from the maximum value occur. A statistical analysis of these results is presented in Table 1.

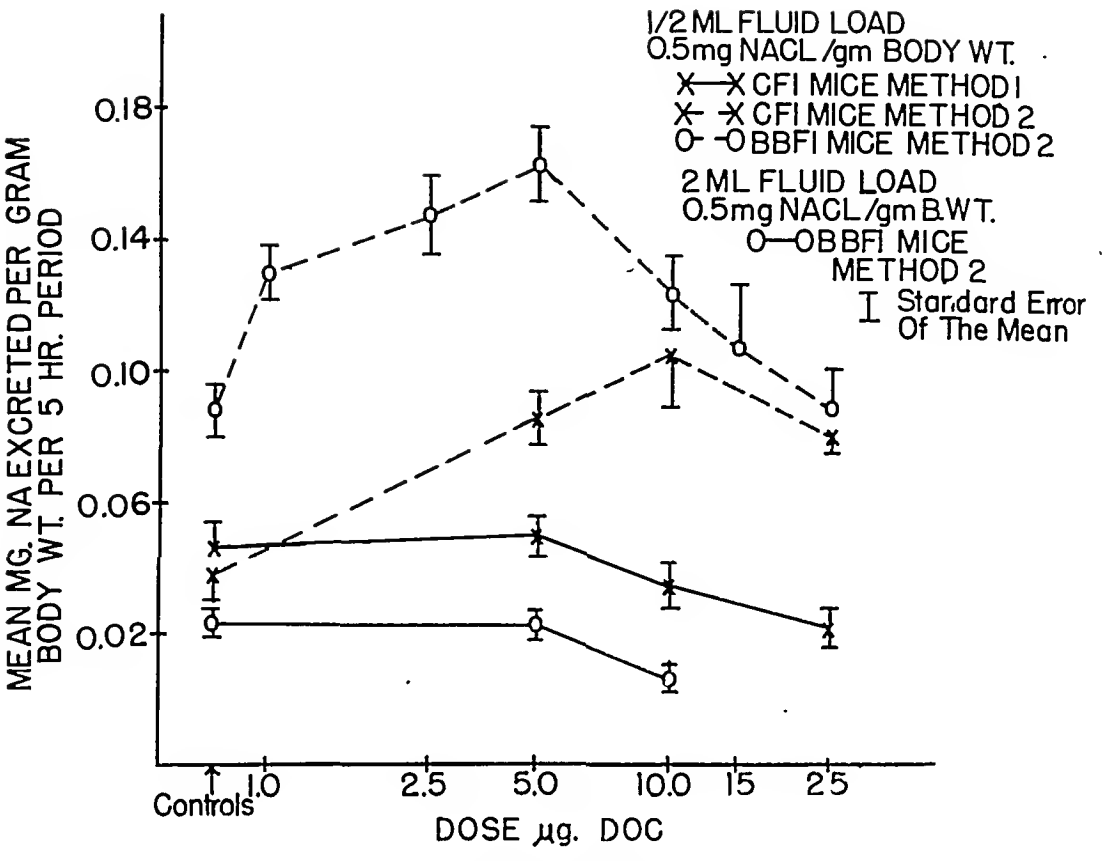

FIG. 2. The comparative effect of desoxycorticosterone on the urinary sodium excretion of strain CF1 mice and strain BBF1 mice. Standard error of the mean indicated in only one direction at times to avoid overlap. Note different scale from figure 1.

Strain BBF1 Mice

To find mice more sensitive to DOC than the CF1 strain, mice of the C57 BBF1 strain were tested. These mice tolerated sodium restriction very badly and when tested with Method 1, the mice were in such poor condition that sodium excretion was always very low regardless of fluid or sodium load used. For this reason studies on this strain are limitd to Method 2. With a sodium load of 0.5 mg. NaCl per gm. body weight and a fluid load

TABLE 1. THE SODIUM EXCRETING AND RETAINING EFFECT OF DESOXYCORTICOSTERONE IN STRAIN CF1 MICE

	Controls	5 µg. DOC	10 µg. DOC	25 µg. DOC
No. of Animals	8	13	11	8
Mean mg. Na Excr. per gm. body wt. per 5 hrs.	0.0386	0.0857	0.1053	0.0806
Stan. error of mean	±0.0087	±0.0086	±0.0150	±0.0059

of 0.5 ml. DOC had a pronounced sodium excreting effect (Fig. 2). Maximal sodium excretion occurred with 5 μg. DOC in contrast to the strain CF1 mice which required 10 μg. DOC for maximal sodium excretion. A nearly linear fall in sodium excretion occurred when plotted against the

TABLE 2. THE SODIUM EXCRETING AND RETAINING EFFECT OF DESOXYCORTICOSTERONE IN STRAIN BBF1 MICE

	Control	1 μg. DOC	2.5 μg.	5.0 μg.	15 μg.	15 μg.	25 μg.
No. of Animals	22	10	8	20	20	8	11
Mean mg. Na excr. per gm. body wt. per 5 hr. period	0.0884	0.1309	0.1480	0.1635	0.1256	0.1083	0.0897
Standard error of mean	±.0088	±.0083	±.0137	±.0102	±.0112	±.0192	±.0138

logarithm of the dose at levels of 10 μg., 15 μg. and 25 μg. doses of DOC in the BBF1 mice. (The numerical values are analyzed in Table 2.) When the same amount of sodium chloride was given in a volume of 2 ml. (Fig. 2) no increased excretion of sodium occurred with small doses of DOC. The promotion of sodium excretion by 5 μg. of DOC decreased markedly with increasing fluid load (Fig. 3).

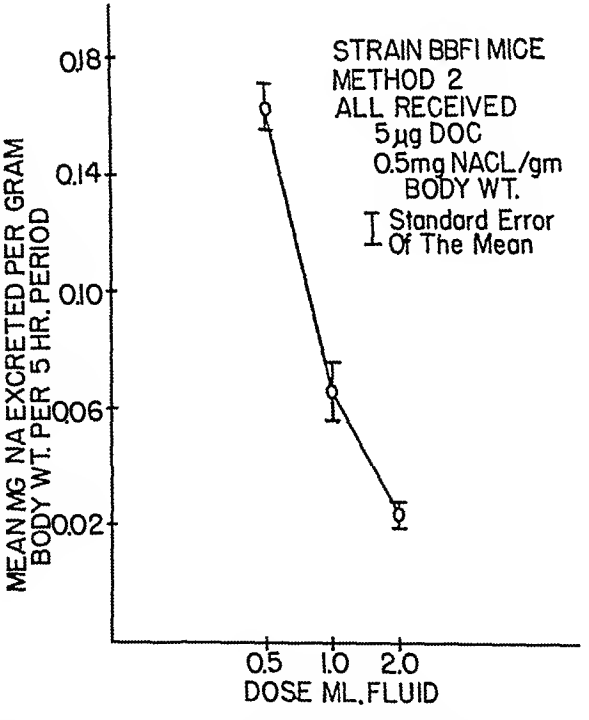

FIG. 3. The effect of increasing fluid load on the maximal urinary sodium excretion of BBF1 mice produced by 5 μg. DOC. Each point represents the mean of from 7 to 20 animals.

To minimize intestinal absorption of exogenous sodium, the drinking water containing sodium chloride was removed 5 hours rather than 3 hours before study. The control sodium excretion was somewhat lower in these experiments with a mean of 0.0601 mg. per gm. body weight per 5 hours, but increased excretion of sodium again occurred with 5 μg. DOC, the mean being 0.1006 mg. per gm. body weight per 5 hours.

Not only did the total amount of sodium but also the concentration of urinary sodium increase in strain BBF1 mice given 5 μg. DOC. Thus at the beginning of the assay 3 hours after removal of saline drinking water, the mean sodium concentration of the urine was 243 m.eq. per kilogram of urine (S.E. = ±41). Three hours after receiving 5 μg. DOC the mean sodium concentration of the urine was 424 m.eq. per kilogram of urine (S.E. = ±45). This difference is significant ($P = 0.01$). Urinary excretion of potassium was not significantly altered by doses of DOC up to 25 μg.

DISCUSSION

Speirs *et al.* (1951) were able to demonstrate that the strain of mice C57 BBF1 was about ten times more sensitive to cortisone as judged by eosinophil response than other strains tested. Our results indicate a comparable sensitivity to DOC in this same strain. These mice require 5 μg. DOC to reach maximal sodium excretion while strain CF1 mice required 10 μg. DOC for maximal sodium excretion. Significant sodium retention occurred in strain BBF1 mice with 10 μg. DOC, but strain CF1 mice required 25 μg. DOC for significant sodium retention. It would appear that mice of the C57 BBF1 strain are two to three times more sensitive to DOC than the mice of the CF1 strain.

The use of the C57 BBF1 strain for the bioassay of DOC seems possible under the conditions specified because of the wide differences in urinary sodium with doses of DOC from 1 μg. to 25 μg. The biphasic character of sodium excretion with DOC would necessitate testing several dosage levels of the material to be assayed in order to locate the value on the ascending or descending slope of the curve of urinary sodium excretion.

The promotion of sodium excretion in our experiments was unexpected. However, desoxycorticosterone has been shown to cause sodium excretion in Cushing's syndrome (Soffer, Gabrilove and Jacobs, 1949) and in normal dogs receiving high salt loads (Green, Farah and Klemperer, 1950). Large doses of desoxycorticosterone glycoside intravenously in humans with presumably intact adrenals causes sodium excretion, but with a fall in urine sodium concentration (Green *et al.*, 1950). The nearly twofold increase in control sodium excretion effected by 5 μg. DOC in adrenalectomized mice of the BBF1 strain could be largely attributed to increased urinary sodium concentration. Gastrointestinal absorption of sodium ap-

pears to contribute little to the sodium diuresis produced by DOC. Sodium excretion with 5 μg. DOC increased approximately to the same degree after animals had been off saline drinking-water for 5 hrs. Increasing the subcutaneous fluid load decreased or abolished the sodium diuresis produced by DOC.

It is known that dogs (Roemmelt, Sartorius and Pitts, 1949) and rats (Friedman, 1948; Lotspeich, 1949) maintain a normal glomerular filtration rate for a period of time after adrenalectomy when adequately hydrated. Animals used in this study had free access to saline solution and were used for a maximum period of seventeen days after adrenalectomy. It is suggested, therefore, that increasing glomerular filtration rate was probably not the mechanism whereby DOC caused increased sodium excretion in the present experiment. Adrenalectomized dogs and patients with Addison's disease have a reduced capacity to excrete sodium under a high sodium load due to increased tubular reabsorption of sodium (Roemmelt, Sartorius and Pitts, 1949; Burnett, 1951). The sodium diuresis in the present experiments would seem to be due to a depression of tubular reabsorption of sodium with small doses of DOC, thus restoring to the adrenalectomized animal a more normal ability to excrete high sodium loads. As the dose of DOC is increased the depression of tubular reabsorption of sodium with small doses of DOC is followed by the more familiar increase in tubular reabsorption of sodium. The data at hand do not permit elucidation of the mechanism of depression of tubular reabsorption of sodium by DOC.

SUMMARY

The optimal conditions for demonstrating the action of DOC in adrenalectomized mice have been studied. A sodium load of 0.5 mg. NaCl per gm. of body weight proved most satisfactory. DOC in small doses promoted sodium excretion under high sodium loads while sodium retention occurred with larger doses of DOC. Adrenalectomized mice of the BBF1 strain are two to three times more sensitive to DOC than mice of CF1 strain.

The increased sodium excretion with small doses of DOC could be accounted for by increased urine concentration of sodium. The sodium diuresis can best be explained by decreased tubular reabsorption.

ACKNOWLEDGMENTS

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EFFECTS OF ANDROGENS ON DEHYDROGENASE SYSTEMS

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WE RECENTLY reported an inhibitory effect on the succinoxidase system of rat liver homogenates by certain water-soluble androgens (Kalman, 1952). There was some evidence in this work that succinic dehydrogenase was the target of the inhibitor. Because different dehydrogenases respond similarly to a number of inhibitors it was decided to try the effect of the androgens on the malic dehydrogenase system. In addition certain observations regarding kinetics of succinoxidase inhibition are included in this report.

METHODS

Adult white rats of the Slonaker-Wistar strain were used. No sex differences in regard to the work reported were noted and males and females were used interchangeably. The animals were killed by decapitation, and appropriate amounts of liver tissue were removed, weighed, and homogenized in cold, distilled water. Homogenates were prepared as described by Potter and Elvehjem (1936). For each enzyme system control runs were made to ensure that optimal amounts of all components were present. Control QO_2 values fell within the range of conventional results. Calculations of oxygen consumption were based on time periods during which control vessels showed essentially linear oxygen uptake. The gas phase for all systems was oxygen. In addition to the 3.0 ml. of reaction mixture, 0.1 ml. of 5% KOH was placed on filter paper in the center well of each vessel.

For the malic dehydrogenase assay the method of Potter (1946) was used. The Warburg vessels contained 0.3 ml. each of 0.5 M malate, 0.5 M glutamate, 1% diphosphopyridine nucleotide (DPN) solution, 0.0004 M cytochrome C, 0.8 ml. of 0.1 M PO_4 buffer at pH 7.4, inhibitor and homogenate in amounts specified below, and distilled water to make 3.0 ml. The DPN was added from the sidearm at the start of the reaction. Nicotinamide was omitted because it did not enhance oxygen consumption over the time periods employed.

The succinoxidase system used was that described in Umbreit's text (1949). The reaction vessel contained 0.3 ml. each of 0.004 M $CaCl_2$, 0.004 M $AlCl_3$, 0.0001 M cytochrome C, 0.5 M succinate, 1.0 ml. of 0.1 M PO_4 buffer at pH 7.4, and inhibitor as described below. A 5% liver homogenate was prepared in the usual way and then centrifuged for 10 minutes at 600×gravity. Homogenate from the supernatant was added to reaction vessels which had been filled with the other reactants and chilled for several minutes.

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The androgens used were sodium androsterone sulfate, sodium testosterone sulfate, and testosterone 17-β diethylaminoethylcarbonate hydrochloride ("soluble" testosterone). These steroids were used in aqueous solutions. The sodium testosterone sulfate and sodium androsterone sulfate are water soluble at pH 7.4. Testosterone 17-β diethylaminoethylcarbonate hydrochloride is insoluble at pH 7.4 (the pH of the reaction vessels) and above, forming a very fine suspension in an aqueous medium. This suspension may be dissolved by bringing the pH back to neutrality or to the acid side. During the course of this work some diethylaminoethanol hydrochloride was made available through the courtesy of Dr. E. Oppenheimer. This compound was tested for possible *in vitro* effect since it forms the major part of the side chain in "soluble" testosterone.

RESULTS

Inhibition of the malic dehydrogenase system by small concentrations of "soluble" testosterone is shown in Table 1. Sodium testosterone sulfate

TABLE 1. EFFECT OF WATER SOLUBLE ANDROGENS ON THE MALIC DEHYDROGENASE SYSTEM OF RAT LIVER

(QO_2 = c.mm. of oxygen uptake per mg. dry tissue per hour at 37.5° C.)

Homogenate mg. dry weight per vessel	Molar concentration		QO_2	Per cent inhibition
	Sodium testosterone sulfate	"Soluble" testosterone		
1.5	—		98	Control
1.5	3.4×10^{-4}		82	16.3
1.5	6.8×10^{-4}		46.8	52.3
1.5	1.0×10^{-3}		26.0	75.0
1.5	1.4×10^{-3}		14.0	86.0
1.5	1.7×10^{-3}		16.0	84.0
3.0		—	105	Control
3.0		1.8×10^{-5}	100	4.8
3.0		2.7×10^{-5}	90	14.2
3.0		3.6×10^{-5}	46	56.0
3.0		4.5×10^{-5}	23	78.0
3.0		5.4×10^{-5}	21	80.0
3.0		6.3×10^{-5}	17	84.0
3.0		7.2×10^{-5}	12	88.5

proved to be much less effective an inhibitor as indicated by the concentrations required. Sodium androsterone sulfate was not available for test on the malic dehydrogenase system.

In Table 2 data are presented which show inhibition of the succinoxidase system by "soluble" testosterone, by sodium androsterone sulfate, and by sodium testosterone sulfate. The inhibitory effect of these compounds as shown by the concentrations used was most pronounced with "soluble" testosterone, less with sodium androsterone sulfate, and least with sodium testosterone sulfate. Diethylaminoethanol hydrochloride does not inhibit succinoxidase in concentrations of 3.5×10^{-5} molar. Such amounts are equivalent to the amount of this compound which is present in the side chain of "soluble" testosterone at concentrations of 9×10^{-5} molar.

TABLE 2. EFFECT OF WATER SOLUBLE ANDROGENS ON THE SUCCINOXIDASE SYSTEM OF RAT LIVER

QO_2 = c.mm. of oxygen uptake per mg. dry tissue per hour at 37.5° C.
All vessels contained 3.0 mg. dry weight of homogenate.
Concentrations of drugs are expressed in final molar concentration

Sodium testosterone sulfate	QO_2	Per cent inhibition	Sodium androsterone sulfate	QO_2	Per cent inhibition
—	76.0	Control	—	86	Control
3.4×10^{-4}	45.0	41.0	9×10^{-5}	81.5	18.9
6.8×10^{-4}	32.0	58.0	1.3×10^{-4}	68.0	26.7
1.0×10^{-3}	13.8	81.5	1.8×10^{-4}	61.0	27.8
1.4×10^{-3}	4.0	95.0	2.3×10^{-4}	60	33.4
1.7×10^{-3}	5.0	94.0	2.7×10^{-4}	57.4	62.3
2.0×10^{-3}	2.6	96.0	3.2×10^{-4}	34.7	92
			3.6×10^{-4}	0	100

"Soluble" testosterone	QO_2	Per cent inhibition	diethylamino-ethanol · HCl	QO_2	Per cent inhibition
—	74	Control	—	87	Control
9×10^{-6}	66	10.8	3.5×10^{-5}	91	—
1.8×10^{-5}	36	51.5	3.5×10^{-5}	94	—
2.7×10^{-5}	14	81.0			
3.6×10^{-5}	8	89.0			
4.5×10^{-5}	3	96.0			
5.4×10^{-5}	2	97.5			

Preliminary results suggest that the water-soluble androgens also have an inhibitory effect on the lactic dehydrogenase system.

The curves of Figure 1 were derived from the data in Table 1 by plotting per cent inhibition of oxygen consumption by "soluble" testosterone for both malic and succinic dehydrogenase systems. Similar curves are obtained for inhibition by the sodium salt of testosterone sulfate of the malic dehydrogenase system and for the inhibition by sodium androsterone sulfate of the succinoxidase system. The inhibition of succinoxidase by sodium testosterone sulfate yields an atypical curve, no explanation for which is at hand.

Incubation time had no apparent effect on the inhibition of succinoxidase in liver homogenate by "soluble" testosterone in an experiment in which succinate was added from the side arms into the main chamber of the Warburg vessels at successive time intervals.

Figure 2 shows the effect of added homogenate on the testosterone inhibition of succinoxidase. Data are plotted according to Ackermann and Potter (1949). At each inhibitor concentration the same amount of enzyme is "bound" regardless of the amount of homogenate present.

DISCUSSION

The most interesting observation noted was the inhibition of the malic dehydrogenase system by "soluble" testosterone and by sodium testosterone sulfate. Erway *et al.* (1947) have reported that sodium androsterone

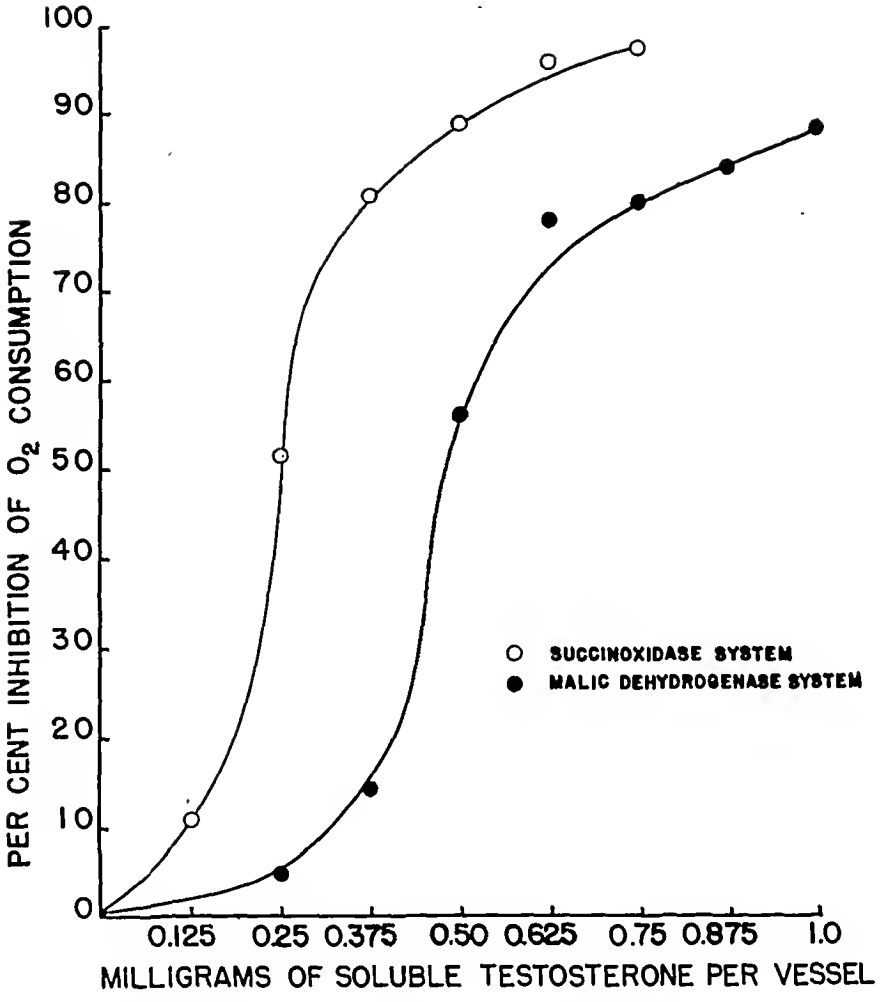

FIG. 1. The effect of concentration of testosterone 17-β diethylaminoethylcarbonate hydrochloride ("soluble" testosterone) on the malic and succinic dehydrogenase systems. 0.125 mg. "soluble" testosterone is equivalent to 9×10^{-5} molar concentration.

sulfate has no significant effect on this enzyme system. They used inhibitor concentrations of 10^{-4} molar.

The inhibition of the succinoxidase and malic dehydrogenase systems by water-soluble steroids used appeared not to fit classical formulations (Lineweaver and Burk 1932). However, the data are compatible with the "irreversible" type of inhibitor described by Ackermann and Potter (1949). A definite reduction in oxygen consumption is effected by a given increment of inhibitor and is not reversed by increasing the amount of enzyme present (Fig. 2). Bain (1949) has demonstrated one instance of this type of inhibition in the effect of certain phosphate esters (diisopropylfluorophosphate, tetraethylpyrophosphate) on cholinesterase. Hayano and Dorfman (1951) showed a similar relationship for the inhibition of a d-amino acid oxidase by desoxycorticosterone.

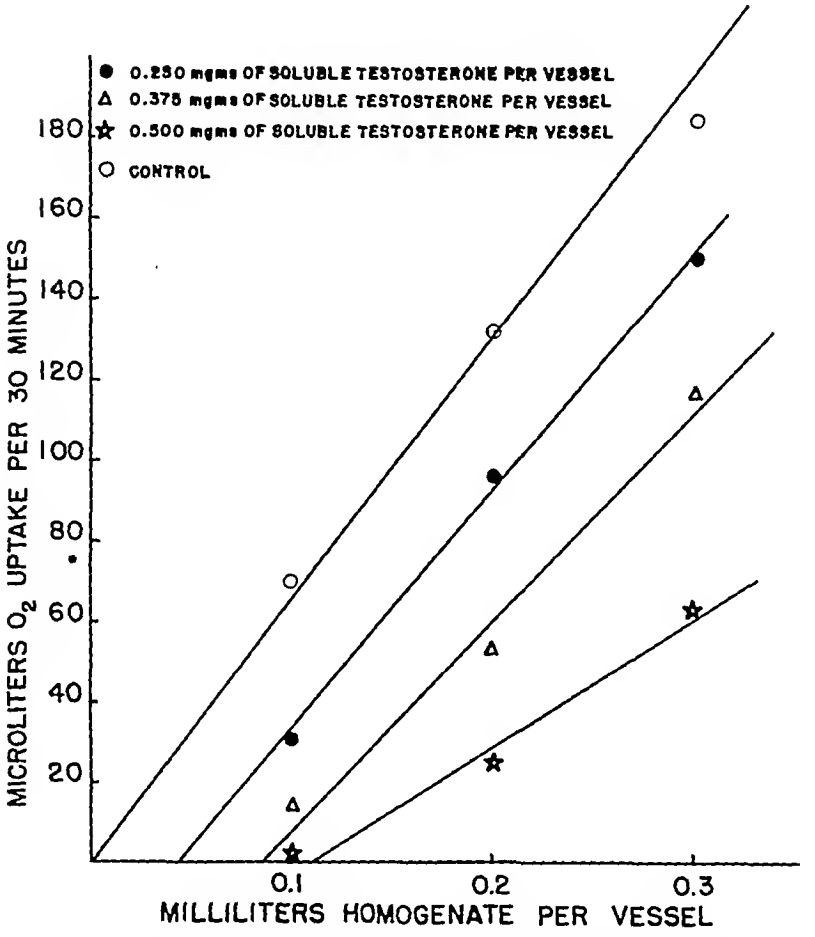

FIG. 2. The effect of increasing amounts of homogenate on the inhibition of the succinoxidase system by different amounts of testosterone 17-β diethylaminoethylcarbonate hydrochloride ("soluble" testosterone). 0.250 mg. soluble testosterone is equivalent to 1.8×10^{-5} molar concentration.

Dr. D. M. Greenberg suggested that the sigmoid shape of the curves relating per cent inhibition to inhibitor concentration (Fig. 1) resembles an adsorption isotherm. Such curves have been obtained, for example, for the amount of water vapor adsorbed on charcoal under conditions of varying pressure (Coolidge 1937). If we consider per cent inhibition analogous to inhibitor uptake, and inhibitor concentration analogous to "pressure," we may postulate that the steroid is adsorbed by the enzyme. The inhibition of saccharase by certain basic dyes has been described by Quastel and Yates (1936). When per cent inhibition was plotted against pH sigmoid curves similar to the ones plotted here were obtained. They suggested that the anion form of saccharase was better able to combine with basic dyes.

The significance of the variation in water solubility of the androgens mentioned above is not apparent. It may be that the long side chain of "soluble" testosterone makes this compound more susceptible to adsorption phenomena.

The site of action of the androgens on the dehydrogenase systems is thought to be the dehydrogenases themselves. Evidence for this has accumulated mainly by inference after other hydrogen carriers were bypassed or proved to be less susceptible to the inhibitors (Eisenberg *et al.*, 1949). A promising method of approach would seem to be fractionation of the components of the Warburg reaction mixtures in an attempt to recover the enzyme-inhibitor complex. Such an approach might also be used to study the enzyme-inhibitor "binding."

SUMMARY

The effects of water-soluble androgens on malic dehydrogenase and succinoxidase of rat liver homogenates were studied using conventional Warburg techniques. These substances were found to inhibit both enzyme systems in a manner suggesting an irreversible combination of enzyme and inhibitor. The data were also suggestive of some type of adsorption phenomenon involving enzyme and steroid.

Acknowledgements

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PATHWAYS OF ELIMINATION OF C^{14}-LABELED THYROXINE IN THE RAT[1]

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INTRODUCTION

TAUROG, Briggs, and Chaikoff (1951) using I^{131}-L-thyroxine demonstrated the presence of an unknown metabolic product, in addition to thyroxine, in the bile of rats. These investigators (1952) then reported that the thyroxine-containing derivative may be a glucuronide, but not a peptide. They further concluded that the conjugate is the primary metabolic product of endogenous thyroxine, and that the bile is the chief pathway of elimination for this hormone.

The present study is concerned with the elimination of DL-thyroxine--1-C^{14} in the bile, urine, and expired air. Further analysis of the bile has indicated that the thyroxine conjugate is not a peptide.

METHODS

DL-thyroxine-1-C^{14} used was that synthesized by Wang, Hummel, and Winnick (1952).[4] Its specific activity was 5.3 μc per mg. In all radioactivity measurements, the observed counts per minute were converted into equivalent μgm. of thyroxine, by reference to the known specific activity of the administered C^{14}-thyroxine. Samples were generally counted to within a probable error of 2 or 3%, and the values corrected for self-absorption. By way of illustration, 0.1 ml. of bile had of the order of 300 counts per minute, or 20 times background, with our counter.

Adult male Sprague-Dawley rats, fed ad libitum, were employed. The bile duct was cannulated with polyethylene tubing (inside diameter, 0.011 inch). When a good rate of flow was observed, the abdomen was closed, and the animal suspended in a restraining harness. Thyroxine was injected subcutaneously at a dose level of 780 μgm. (one μM) per kilo body weight. Bile samples were collected in 2 ml. portions over a 12 to 14-hour

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[4] The C^{14} for the synthesis of the thyroxine was obtained on allocation from the U. S. Atomic Energy Commission.

period. The radioactivity of the whole bile was determined by drying 0.1 ml. aliquots on aluminum plates, and counted in a thin mica window Geiger counter.

Paper chromatograms were performed as described by Taurog and co-workers (1951). Aliquots of 0.1 ml. bile were chromatographed immediately after the bile was collected. After the chromatogram was completed, the paper was cut into 1 cm. strips, and each strip extracted three times with 0.5 ml. of 0.01N NaOH. The extracts were air dried on aluminum plates for counting. Approximately 53% of the added thyroxine activity was recovered from the paper chromatograms.

The whole bile was subjected to the Van Slyke ninhydrin-carbon dioxide method (1941) which indicates the presence of free carboxyl and amino groups in amino acids. These determinations were performed at pH 2.5 on 0.1 ml. bile samples. The $BaCO_3$ was collected and counted as described by Winnick (1950).

A Benedict closed circuit metabolic apparatus was employed for the collection of the expired $C^{14}O_2$. The latter was removed from the expired air by bubbling the circulating gases through a 1N NaOH solution which was replaced at 2 or 3-hour intervals. Aliquots of the NaOH solutions were treated with excess $BaCl_2$, the precipitated $BaCO_3$ washed, dried, and plated for counting.

The concentration of C^{14} activity in the urine was determined on 0.1 ml. aliquots of samples, obtained at the various time intervals. The urine, which was collected from rats placed in metabolic cages, was plated as described for the bile.

RESULTS AND DISCUSSION

Figure 1 shows that the C^{14} of carboxyl-labeled DL-thyroxine is most rapidly eliminated by way of the bile. About 20% of the radioactivity was found in the bile in 12 hours. Almost identical results were obtained in 5 repeated experiments. Taurog and co-workers (1951) give a comparable value of 28% eliminated in 12 hours, for a subcutaneous injection of 330 μgm. of I^{131}-labeled L-thyroxine per kilo.

The appearance of 10% of the C^{14} in respiratory carbon dioxide in 12 hours indicates that the alanine portion of the thyroxine molecule is catab-

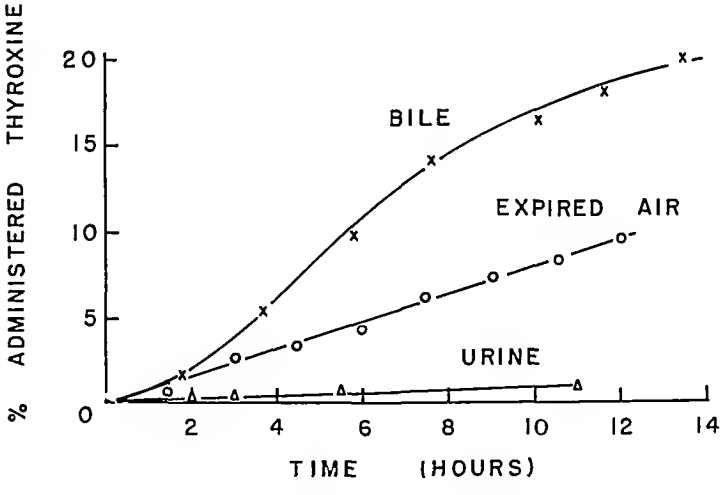

FIG. 1. Elimination of C^{14}-carboxyl labeled thyroxine by different routes.

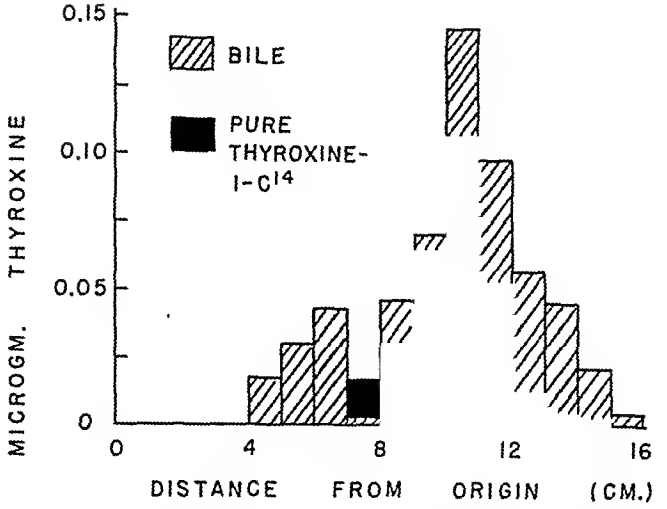

FIG. 2. Chromatogram of 0.1 ml. aliquot of a 2 ml. sample of bile, taken 1.8 to 3.8 hours after administering thyroxine-1-C^{14}.

olized. Essentially the same rate of elimination of $C^{14}O_2$ was obtained in 5 other experiments.

A relatively low C^{14} concentration, about 1% of the total dose, appeared in the urine in 12 hours. The average value was 1.2% for 10 such experiments. This radioactivity may represent, in part, urea derived from $C^{14}O_2$ by way of the ornithine cycle. Gross and Leblond (1949) reported a urinary excretion of about 10% of intravenously administered I^{131}-thyroxine in rats after 24 hours. Most of the I^{131} could not be extracted from the urine by butanol, and was referred to as "non-thyroxine" iodine.

The results of a paper chromatogram performed on bile from a rat given C^{14}-thyroxine is shown in Figure 2. The small peak accounted for 10%, and the larger, more rapidly moving peak 53%, of the total C^{14} in the bile sample. The position of the larger peak is identical with that of thyroxine. These results show that, at the 780 μgm./kilo dosage, free thyroxine predominates in the bile. This conclusion is in accord with the finding of Taurog and co-workers (1952) that large doses of thyroxine-I^{131} are conjugated to only a very limited extent in the rat. Because of the limitations imposed by the specific radioactivity of our thyroxine-1-C^{14}, it was not possible to employ the latter at physiological levels.

The data in Figure 3 indicate that virtually all of the C^{14} of the bile is released by ninhydrin treatment. The agreements are within the limits of experimental error. Van Slyke, Dillon, MacFadyen, and Hamilton (1941) have shown that amino acids are decarboxylated by ninhydrin only when both amino and carboxyl groups are in the uncombined state. Although

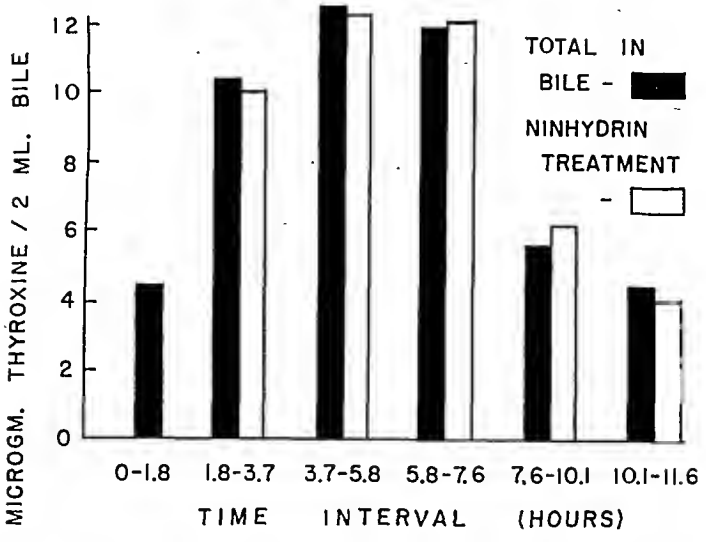

FIG. 3. Thyroxine excretion, as measured by total C^{14} in bile, and by $C^{14}O_2$ released from bile by ninhydrin.

chromatography indicates that a minor portion of the thyroxine exists in conjugated form, this fraction may be bound through the phenolic hydroxyl group, and hence would still react with ninhydrin.

SUMMARY

The various routes of elimination of thyroxine-1-C^{14} in normal adult rats were investigated. Labeled thyroxine was administered subcutaneously at a level of 780 μgm./kilo. The bile was the major route of excretion, and contained about 20% of the C^{14} after 12 hours. Approximately 10% of the C^{14} appeared in the expired CO_2, and 1% in the urine, in this period.

Paper chromatographic analysis of a bile sample taken between 2 and 4 hours, revealed the presence of a small secondary component, in addition to a large thyroxine peak. Treatment of bile samples with ninhydrin resulted in a quantitative release of C^{14} as $C^{14}O_2$. This finding indicates that both amino and carboxyl groups of the thyroxine in the bile are in an uncombined state.

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ESTIMATED DURATION OF THE SPONTANEOUS ACTIVATION WHICH CAUSES RELEASE OF OVULATING HORMONE FROM THE RAT HYPOPHYSIS[1]

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INTRODUCTION

NEUROGENIC activation of the rabbit adenohypophysis causing discharge of ovulating hormone is now considered to be accomplished within about one minute after coitus (Sawyer, Markee *et al.*, 1947, 1949). Actual discharge into the bloodstream of hormone sufficient for ovulation requires approximately one hour in that species (Fee and Parkes, 1929).

Whereas, in spontaneously ovulating animals, discharge of the hormone was long thought to be invoked by direct action of ovarian steroids on the hypophysis, there is now strong evidence that this effect is mediated by the hypothalamus (Sawyer, Everett and Markee, 1949; Everett and Sawyer, 1949). Activation of the hypophysis in cyclic rats occurs within a restricted period of time during the day of proestrus (Everett, Sawyer and Markee, 1949). In our colony this critical period is between 2 and 4 P.M. for the great majority of animals (4-day cycle), about 9 to 11 hours before ovulation. The injection of certain blocking agents at 2 P.M. or earlier will prevent ovulation, while their injection at 4 P.M. will not usually interfere with it.

Studies with the rabbit have demonstrated that antiadrenergic agents of the β-haloalkylamine series (Dibenamine and SKF-501) and certain anticholinergic agents (atropine and Banthine) will block the reflexogenous stimulus to the hypophysis when injected intravenously within a fraction of a minute after coitus (Sawyer, Markee *et al.*, 1947, 1949, 1950, 1951). These agents are similarly effective in blocking the "spontaneous" activation of the hypophysis in cyclic rats. In addition, the barbiturates have been effective in the latter species, apparently because they can be intro-

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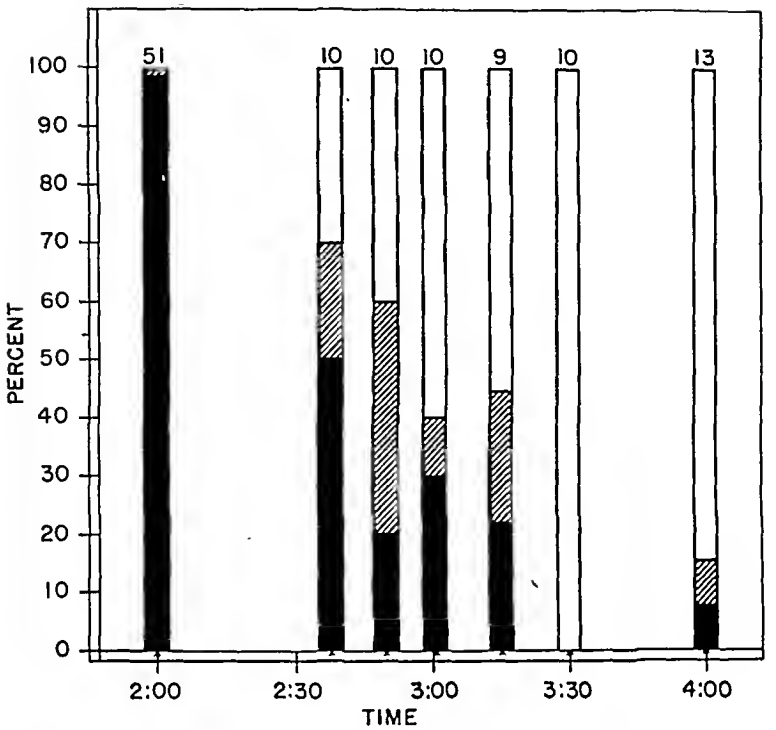

FIG. 1. Results of injection of atropine on the day of proestrus at different times between 2:00 and 4:00 P.M. Solid black: complete blockade. Cross-hatching: partial blockade. White: complete ovulation. The number above each bar indicates the respective number of rats in that time-group. The 2:00 and 4:00 P.M. data are from previous work with atropine and Nembutal (From Everett, 1952, by courtesy of the Ciba Foundation).

gression, for the following considerations. During the first 50 minutes 65% of 20 rats had begun activation, while during the succeeding 40 minutes the percentage increased to only 83% of the remaining 29 rats. Even as late as 4 P.M. 1 case of complete blockade and 1 of partial blockade were encountered. While the former may have been a rare animal which whould have failed to ovulate without atropine, the latter was not. In her case activation must have begun only a short time before injection. It seems probable that an average of the combined 3:30 and 4 P.M. data gives a more nearly true value than either alone.

The occurrence of 9 cases of partial activation, rather uniformly distributed among the 2:40–3:15 time-groups, confirms the earlier impression that the activation stimulus is not momentary. Such a conclusion, however, requires that under the conditions of administration of atropine in massive doses by the subcutaneous route the drug abruptly assumes full blocking concentration at its locus of action, with no significantly prolonged interval during which it would only moderately diminish the

strength of stimulus. The following control experiments seem to give this assurance.

A threshold dose for subcutaneous injection was arrived at empirically: 230 mg./kg. (23 mg. atropine sulphate per ml. of physiological saline). This amount was administered to each of 15 proestrous 4-day cyclic rats at 2:00 P.M. At autopsy next morning the tubes were searched for ova and the excised ovaries were critically examined under the dissecting microscope. In the absence of tubal ova all follicles showing any degree of hyperemia were dissected out with iridectomy scissors and were individually crushed in a small drop of saline between slide and coverslip for microscopic examination. Eight of the 15 rats were judged to have been completely blocked, while 6 rats had ovulated completely. In the remaining animal 3 follicles were markedly hyperemic and when these were crushed prominent corona radiata were seen. Maturation of the ova was in progress and the first polar body was seen in one instance. Partial activation in this one exceptional animal may be assigned to an unusually early beginning of hypophyseal activation, similar to the one case from the Nembutal series included in the 2:00 P.M. group in figure 1. The absence of *numerous* examples of partial activation in the present series, intergrading between complete blockade and complete ovulation, attests an essentially all-or-none effect of atropine at threshold level and signifies that no prolonged interval of partial effectiveness of atropine precedes the time at which full blockade is accomplished.

The second series of control experiments approached the question in another way. If the cases of partial blockade in the definitive series (Fig. 1) were mainly representative of a prolonged interval of partial effectiveness of atropine associated with slow absorption from the subcutaneous site, the intravenous injection of the maximum amount tolerated, immediately before the standard subcutaneous dose at some time during the critical period, should have an all-or-none effect. Most female rats of this strain will tolerate intravenous injection of 70 mg./kg. of atropine sulphate (35 mg./ml. in Ringer-Locke solution). This amount was now given to each of 10 proestrous 4-day cyclic rats at one or another time between 2:30 and 3:20 P.M., the entire amount being introduced within 70 to 80 seconds. (Two additional rats died within a few minutes after injection.) Subcutaneous injection of the 700 mg./kg. dose was completed within a minute afterward. Marked tremors were noted before the intravenous injections were complete and involuntary contractions of various muscle groups were regularly seen when the animal was released. It is significant that in 3 of the 10 rats partial blockade was encountered. At autopsy on the morning after injection, 2 of these had a single follicle showing corona formation and maturation changes, while the third animal had shed 2

ova and most of her unruptured follicles were markedly hyperemic. This considerable number of animals showing partial effects supports the interpretation that they arise from abrupt interruption of a lengthy process of hypophyseal activation.

Inasmuch as the chance of interrupting a progressive process is a direct function of its duration, the latter can be estimated in the present instance from the percentage partially activated. If regression of percentage blocked were linear with respect to time, a direct transformation could be made from percentage partially activated to minutes elapsed. Inasmuch

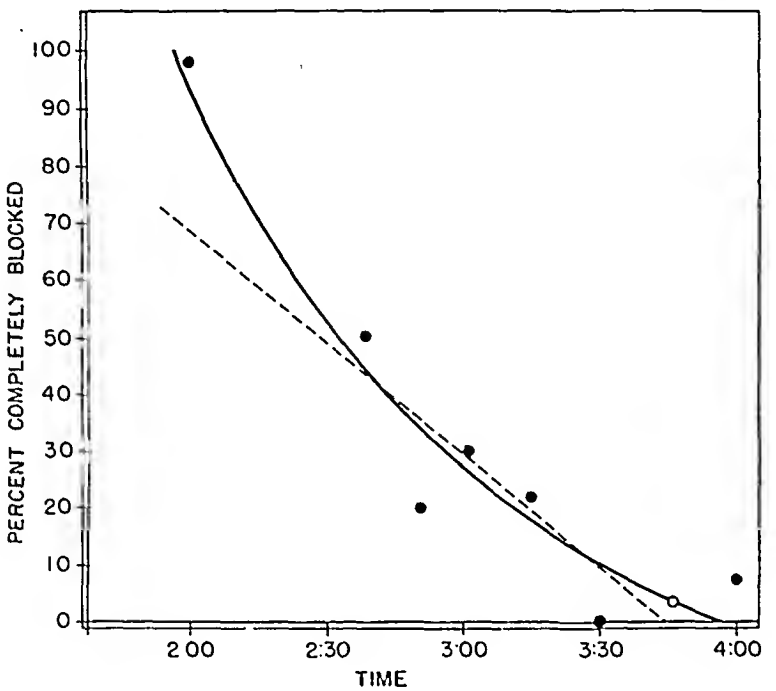

FIG. 2. Diminishing frequency of complete blockade after 2:00 P.M., inversely measuring the beginning of hypophyseal activation. The logarithmic curve is mathematically fitted to the percentages represented by solid circles (the open circle represents the average of the 3:30 and 4:00 P.M. values). The straight (broken) line is approximated to the curve between the 2:40 and 3:30 points (see text).

as the regression is curvilinear, however, certain approximations are required to allow such transformation.

In Figure 2 a logarithmic curve is mathematically fitted to the points representing percentages completely blocked. A straight (broken) line is approximated to the logarithmic curve between the 2:40 and 3:30 points. The slope of this line is such that 10% on the y axis corresponds to 15.3 minutes on the x axis. In terms of this slope, the 9 cases (18.4%) of partial activation among 49 rats of the 2:40–3:30 groups correspond to 28 minutes on the time axis.

This value, however, is admittedly a very rough approximation and has little significance in itself. An important source of error is chance variation in the percentage partially activated. By use of Fisher's table VIII$_1$ (Fisher and Yates, 1949), entered with a = 9 (a/N = 9/49 = 0.184) the lower limits of expectation were obtained.[3] At probability levels of 0.025 and 0.005 these limits were 10% and 8%, respectively, corresponding to 13 and 10 minutes (fig. 3). Thus, the probability is high (0.975) that duration of stimulation is greater than 13 minutes, based on the slope of the straight line from figure 2. An extremely unlikely lower limit may be calculated

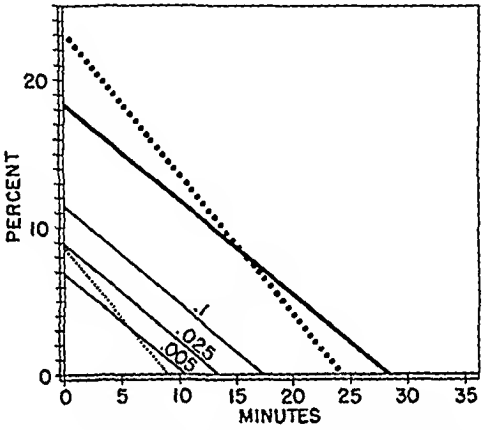

FIG. 3. Relation between percentage partially blocked and time. The heavy solid line has the same slope as the broken line in figure 2. Lower limits of expectation at three levels of probability are represented by the narrow solid lines. Significance of the dotted lines is given in the text.

by disregarding the evident curvilinearity of regression and employing as the steepest plausible slope a straight line drawn between the 2:40 P.M. and 3:30 P.M. values. Since on this assumption all activation would be completed before 3:30, this group is excluded in calculating the percentage partially activated and Fisher's table VIII$_1$ is entered with a = 9 (a/N = 9/39 = 0.231). At the probability level of 0.005 this gives a lower limit of 9 minutes (Fig. 3, lower dotted line).

The calculated limits do not take into consideration the numerous cases of partial activation in other comparable experiments, e.g. the progesterone-atropine series cited earlier (Everett and Sawyer, 1949). Among 78 appropriate examples from such experiments, 20 (24%) were classed as partial activation. Thus the frequency of partial activation in the present series appears to be more truly representative than the cal-

[3] The upper limit was given directly by the fact that 3 of 10 rats in the 2:40 group were fully activated. Their periods of activation must have been less than 40 minutes.

culations imply. Consequently it seems reasonable to judge that in any given rat the duration of stimulus is within the range of 20 to 35 minutes.

DISCUSSION

The results confirm the earlier conclusion (Everett, Sawyer and Markee, 1949; Everett and Sawyer, 1950) that in 4-day cyclic rats of our colony a specific neurogenic stimulus, essential to the discharge of ovulating hormone, passes to the adenohypophysis within restricted, predictable time limits on the afternoon of the day of proestrus. In addition, it is now shown that this stimulus in any one individual is considerably shorter than the 2-hour critical period (2 to 4 P.M.) previously defined. On the other hand, the stimulus lasts much longer than the reflexogenous one in the rabbit. In that species the cholinergic (atropine-sensitive) phase is thought to be completed early in the first minute post-coitum, while the adrenergic (Dibenamine-sensitive) phase is only slightly longer, approximately 1 minute (Sawyer, Markee and Hollinshead, 1947; Sawyer, Markee and Townsend, 1949; Sawyer, Markee and Everett, 1950). In the rat the cholinergic phase of the stimulation process lasts for over 9 minutes, more probably between 20 and 35 minutes and certainly less than 40 minutes.

It seems likely that in the rat the central (Nembutal-sensitive) phase and the cholinergic and adrenergic phases of the stimulation process take place almost concurrently. Time differences of the order found in rabbits would be obscured by the far greater over-all duration.

A quantitative relationship apparently exists between length of time of hypophyseal stimulation and the amount of ovulating hormone released. Such an interpretation rests on the finding in rabbits that none of the blocking agents interferes directly with passage of ovulating hormone from the hypophyseal cells into circulation (Sawyer, *et al.*, loc. cit.). Since a species difference in this respect is unlikely, the numerous cases of partial blockade in the present series should then represent incomplete release resulting from foreshortened stimulation. Indeed, if the normal period of stimulation approximates half an hour, stimulation and release may proceed concurrently. While in rabbits about an hour is required for release (Fee and Parkes, 1929), the time in rats could easily be somewhat shorter.

It should be noted that in at least two of the species in which ovulation depends on copulation, relatively prolonged stimulation from lengthy or repeated copulation is indicated (*ferret,* Hill and Parkes, 1932; *mole shrew,* Pearson, 1944). In the mole shrew 1 to 3 copulations per day are said to be ineffective, whereas ovulation regularly follows when the number is increased to 10 to 15 for another two days. In contrasting the rabbit and the ferret, Hill and Parkes spoke of the "trigger" mechanism characteristic of the former species, although its momentary nature had not been eluci-

dated at that time. It seems probable that in spontaneous ovulators and reflex ovulators alike there may be rather wide species variation in the duration of the stimulation process.

There are some indications that under normal circumstances the rat hypophysis continues to be stimulated for a considerably longer period than that which is just sufficient to release the minimal amount of gonadotrophin capable of ovulating all the follicles. Everett, Sawyer and Markee (1949) noted in the original 4 P.M.-atropine series that while all animals ovulated, the corpora lutea from the preceding cycle failed to store visible cholesterol within the usual time. Such cholesterol storage has been shown to be a function of LH (Everett, 1947). Another function of LH is seen in the depletion of cholesterol in the interstitial tissue (Claesson and Hillarp, 1947; Everett, 1949). In our 4-day cyclic rats such depletion is normally so extreme on the morning after ovulation that the ovarian interstitium presents a dull, watery appearance. In the present experiments this extreme depletion was noted in only 24 of 35 rats (69%) in the "fully ovulated" category. It seems likely, therefore, that the estimate of 20 to 35 minutes simply represents the minimal stimulation necessary for ovulation and that the normal stimulation lasts for some longer time, causing relase of an excess of gonadotrophin. This factor of insurance may be reflected not only in the lipid changes in corpora lutea and interstitial tissue, but also in the uniformity of ovulation time observed in these animals.

SUMMARY

Previous study of 4-day cyclic rats (inbred Vanderbilt strain) has defined a 2-hour interval during proestrus, within which neurogenic ovulatory activation of the adenohypophysis takes place. In the present study, 49 such rats were injected with the standard blocking dose of atropine at different times during that interval and were autopsied the next morning. From the results, in particular from the relative frequency of partial blockade, it is estimated that to release sufficient gonadotrophin for complete ovulation the required duration of stimulus is between 20 and 35 minutes. Collateral evidence suggests that the stimulus may normally continue somewhat longer, serving to release an excess of ovulating hormone.

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